AD_____

GRANT NUMBER DAMD17-94-J-4157

TITLE: A Novel Model System to Examine Agents Used in Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Jennifer Coll
Dr. Linda H. Malkas

CONTRACTING ORGANIZATION: University of Maryland
School of Medicine
Baltimore, Maryland 21201

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE
A Novel Model System to Examine Agents Used in Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-94-J-4157

6. AUTHOR(S)
Jennifer Coll
Dr. Linda H. Malkas

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland School of Medicine
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

We have previously described the isolation and characterization of an intact multiprotein complex for DNA synthesis, designated the DNA synthesome, from human breast cancer cells and biopsied human breast tumor tissue. In the presence of the viral large T-antigen and simian virus 40 (SV40) origin sequences, the DNA synthesome fully supports semiconservative SV40 DNA replication *in vitro*. We have shown that the proteins and enzymes constituting the breast cell DNA synthesome include: DNA polymerases α, δ and ε, DNA primase, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C) and replication protein A (RP-A). We report here that DNA ligase I and poly(ADP-ribose)polymerase (PARP) are also components of the DNA synthesome. Moreover, in this report, we describe the results of experiments designed to map the protein-protein interactions between several of the components of the breast cell DNA synthesome. Overall, our data strongly suggest that breast cells utilize an asymmetric multiprotein complex to replicate DNA.

Furthermore, as the DNA synthesome represents the intact breast cell's DNA replication machinery, it may serve as a novel model for examining the mechanisms of action of anti-breast cancer agents that target the DNA synthetic process. In this report, we present data for the first time that indicate that the DNA synthesome serves as a powerful model for studying the actions of irenotecan (CPT-11) and etoposide (VP-16)--two agents that trap nuclear topoisomerases I and II, respectively, in ternary cleavable complexes. We found a close correlation between the IC_{50} values for —)

14. SUBJECT TERMS	15. NUMBER OF PAGES
Human Breast Cells, Cell Proliferation, Multiprotein Complex, DNA, Anticancer Drug, in vitro, Anticancer Agent, Breast Cancer	86
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

the inhibition of intact cell and DNA synthesome-mediated *in vitro* DNA replication by CPT-11(SN-38) (2.0 μM and 0.2 μM, respectively) and VP-16 (2.0 μM and 0.5 μM, respectively). Additionally, we found that similar concentrations of SN-38 and VP-16, 0.5 μM each, inhibited topoisomerase I and II enzymatic activities by 50% as well as produced significant levels of cleavable complexes. Consistent with these findings, alkaline agarose gel electrophoretic analysis of the DNA products synthesized *in vitro* indicate that SN-38 and VP-16 (0.2 μM and 0.5 μM, respectively) strongly inhibit the elongation of nascent DNA molecules by the DNA synthesome. Ultimately, utilization of the breast cell DNA synthesome as a model for studying the mechanisms of action of CPT-11 and VP-16 may aid the development of improved analogues of the agents for breast cancer treatment.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JC Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JC In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jennifer M. Call 7/31/97

PI - Signature Date

Table of Contents

THE PROTEIN-PROTEIN INTERACTIONS WITHIN THE CORE COMPONENT OF THE BREAST CELL DNA SYNTHESOME*

Jennifer M. Coll[a], Robert J. Hickey[b-e], Erica A. Cronkey[a], Hai-Yan Jiang[a], Lauren Schnaper[f,g], Lahja Uitto[h], Juhani E. Syvaoja[h] and Linda H. Malkas[a-e+]

[a]Department of Pharmacology and Experimental Therapeutics, [c]Program in Molecular and Cellular Biology, [d]Program in Oncology, [e]Program in Toxicology, [f]Department of Surgery, University of Maryland at Baltimore, School of Medicine, Baltimore, MD 21201; [b]Department of Oral and Craniofacial Biology, University of Maryland School of Dentistry, Baltimore, MD 21201; [g]Breast Evaluation and Treatment Center, Greater Baltimore Medical Center, Baltimore, MD 21240, [h]Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN 90570 Oulu, Finland.

Running Title: Breast Cancer Cells Contain a Multiprotein Complex for DNA Replication

Key Words: breast cancer; DNA replication; multiprotein complex; DNA polymerase; DNA synthesome

ABSTRACT

We have previously described the isolation and characterization of an intact multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer cells and biopsied human breast tumor tissue (Coll et al., 1996, Oncology Research 8:435-447). The purified DNA synthesome was observed to fully support DNA replication *in vitro.* We had also proposed a model for the breast cell DNA synthesome, in which DNA polymerases α, δ and ε, DNA primase and replication factor C (RF-C) represent the core component, or tightly associated, proteins of the complex. This model was based on the observed fractionation, chromatographic and sedimentation profiles for these proteins. We report here that poly(ADP-ribose)polymerase (PARP) and DNA ligase I are also members of the breast cell DNA synthesome core component. More importantly, in this report we present the results of co-immunoprecipitation studies that were designed to map the protein-protein interactions between several members of the core component of the DNA synthesome. Consistent with our proposed model for the breast cell DNA synthesome, our data indicate that DNA polymerases α and δ, DNA primase, RF-C as well as proliferating cell nuclear antigen (PCNA) tightly associate with each other in the complex, whereas, DNA polymerase ε, PARP and several other components were found to interact with only PCNA or DNA polymerase α. The association of PARP with the synthesome core suggests that this protein may serve a regulatory function in the complex. Also, the co-immunoprecipitation studies suggest that polymerases α, δ and ε participate in the replication of breast cell DNA. To our knowledge this is the first report ever to describe the direct physical association of polypeptides constituting the intact human breast cell DNA replication apparatus.

Title Footnote:

*This work was supported in part by research awards CA57350, CA65754 and CA73060 from the National Institutes of Health/National Cancer Institute to LHM. Also a University of Maryland School of Medicine Designated Research Initiation Fund award to LHM and a University of Maryland Cancer Center Research Award to RJH supported this work. JMC is a recipient of a U.S. Army Breast Cancer Research Program Predoctoral Fellowship.

+ **Author: Linda H. Malkas,** author to whom correspondence should be addressed

Address: University of Maryland School of Medicine
(a) Department of Pharmacology and Experimental Therapeutics
(c) Program in Molecular and Cellular Biology
(d) Program in Oncology
(e) Program in Toxicology
685 W. Baltimore St.
Baltimore, MD 21201

Tel: (410) 706-2313 or 1798
Fax: (410) 706-0032

Author: Jennifer M. Coll

Address: University of Maryland at Baltimore
(a) Department of Pharmacology and Experimental Therapeutics

Author: Robert J. Hickey

Address: University of Maryland School of Dentistry
(b) Department of Oral and Craniofacial Biology
(c) Program in Molecular and Cellular Biology
(d) Program in Oncology
(e) Program in Toxicology

Author: Erica A. Cronkey

Address: University of Maryland at Baltimore
(a) Departrnent of Pharmacology and Experimental Therapeutics

Author: Hai-Yan Jiang

Address: University of Maryland at Baltimore
(a) Department of Pharmacology and Experimental Therapeutics

Author: Lauren Schnaper

Address: Greater Baltimore Medical Center
(g) Breast Evaluation and Treatment Center
(t) Department of Surgery

Author: Lahja Uitto

Address: University of Oulu
(h) Biocenter Oulu and Department of Biochemistry

Author: Juhani E. Syvaoja

Address: University of Oulu
(h) Biocenter Oulu and Department of Biochemistry

INTRODUCTION

Breast cancer progression is associated with the occurrence of numerous genetic alterations that favor the uncontrolled proliferation of mammary cancer cells (1). Inactivating mutations in the *p53* or *cyclin D* genes, for example, abrogate cell cycle check points and lead to increased cellular proliferation rates and the accumulation of extensive DNA damage (2). Among the markers used in breast carcinoma, a high tumor proliferation rate correlates closely with poor prognosis for overall patient survival (3). Therefore, elucidating the molecular mechanisms of breast cell DNA replication could be critical to the development of improved anti-breast cancer therapies.

To date, several reports have described the isolation of large macromolecular complexes of replication-essential polypeptides from the extracts of eukaryotic cells (4,5,6,7). For example, a megacomplex of enzymes involved in DNTP synthesis and DNA polymerization, known as replitase, has been purified from Chinese hamster embryo fibroblast cells (CHEF/18) (5). Additionally, the isolation of 100-150S megacomplexes from regenerating rat liver that contain DNA polymerase α-primase and associate with the nuclear matrix has been reported (7). Our laboratory was the first to isolate and characterize a fully functional multiprotein complex for DNA synthesis, designated the DNA synthesome, from human cervical cancer (HeLa) cells as well as from murine mammary carcinoma (FM3A) and human leukemia (HL60) cells (8-11). We have also recently reported that the DNA synthesome also can be isolated successfully from human breast cancer cells and breast tumor tissue (12). Importantly, in all cases the DNA synthesome was shown to fully support semi-conservative papovavirus DNA replication *in vitro,* in the presence of the viral large T-antigen and plasmid DNA containing papovavirus origin

sequences. Since papovaviruses are extensively dependent on the host cell DNA replication apparatus for their own viral genome synthesis, this indicates that the DNA synthesome must also function in mammalian cell DNA replication.

We have also demonstrated that the integrity and function of this multiprotein complex is maintained after its treatment with high-salt, non-ionic detergents, RNase, DNase, anion exchange chromatography, sedimentation through glycerol or sucrose gradients and electrophoresis through native polyacrylamide gels (8-13). These results indicate that the components of the DNA synthesome associate with each other independent of non-specific interactions with other cellular macromolecules.

Several proteins have been shown to be required for eukaryotic DNA synthesis *in vitro* (14,15), and these proteins have been shown to co-purify with the DNA synthesome (8-12) and include: DNA polymerases α, δ and ε, proliferating cell nuclear antigen (PCNA), RF-C, RP-A, topoisomerases I and II, and DNA primase. A model representing the synthesome has been proposed based on the observed fractionation, chromatographic and sedimentation profiles of the proteins that co-purified with the complex (9-12). The synthesome's core component was proposed to include the replication elongation proteins DNA primase, RF-C and DNA polymerases α, δ and ε due to their exclusive partitioning with one another during the course of synthesome purification. In this report we describe the first experimental evidence that indicates a direct physical association between several protein members of the proposed breast cell synthesome core component. This is important since models for the organization of DNA synthetic proteins into replicating machines have been proposed (16); however, it is still unclear how these replication factors associate with one another to facilitate the efficient and coordinated

process of DNA synthesis in mammalian cells. This report describes studies aimed at beginning to address this problem.

MATERIALS AND METHODS

Cell Culture. Suspension cultures of MDA MB-468 human breast cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and fetal bovine serum. Exponentially growing cells (5x 10^5 cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS) (17): 20 mM Na_2HPO_4, 0. 15 mM NaCl, 2.7 mM KCI, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation.

Isolation and purification of the DNA synthesome from MDA MB-468 breast cancer cells and human breast tumor tissue. The DNA synthesome was isolated from MDA MB-468 cells (20 g) and an infiltrating type ductal carcinoma of the female mammary gland according to our previously published procedures (12). We have shown previously that the NE/S-3, P4 and Q-Sepharose peak fractions contain the replication-competent DNA synthesome, whereas the S-4 and Q-Sepharose anion-exchange column flow-through fractions do not contain the complex.

***In vitro* SV40 DNA replication assay**. *In vitro* DNA replication assays were performed according to previously published procedures (18). One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol of dNMP into newly synthesized DNA per 2h under the standard assay conditions.

Purification of SV40 large T-antigen. SV40 large T-antigen was purified from 293 cells infected with a recombinant adenovirus vector, Ad-SVR284, as detailed elsewhere (19).

Enzyme Assays. DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures (20,21). One unit of DNA polymerase α activity is equivalent to the incorporation of 1nmol of [^{3}H]-TMP into DNA per hour at 37°C. **DNA primase** activity with single-stranded poly(dt) template DNA was also assayed according to previously published procedures (21). One unit of DNA primase activity is equal to the incorporation of 1 nmol of [^{3}H]-dAMP into DNA per hour at 30°C.

Co-immunoprecipitation of the core protein members of the DNA synthesome by PCNA and DNA polymerases α **and** δ **antibodies.** One hundred μg of the Q-Sepharose peak fraction from MDA MB-468 breast cancer cells or fifty μg of the P-4 fraction from human breast tumor tissue was precleared with either protein A or G-conjugated agarose beads for 10 minutes at 4°C. The precleared protein fractions were next incubated overnight at 4°C with monoclonal antibodies directed against PCNA (mAb-10; 2.5 μg/reaction), DNA polymerase δ (mAb-11; 80 μl tissue culture supernatant/reaction) or DNA polymerase α (SJK 132-20 (39); 20 μg purified ascites/reaction). Eighty μl of a 10% suspension of protein A or G conjugated-agarose beads, resuspended in buffer 1 (500mM NaCl, 25mM Tris-HCl (pH 7.5), 5mM EDTA (pH 7.5) and 1% Triton X-100), were then added to the respective reactions and the incubations continued for an additional 2 hours at 4°C. The reaction mixtures were then centrifuged at 3500 rpm for five minutes at 4°C, and the supernatant and pellet fractions were reserved. Subsequently, the pellet fraction was washed three times with buffer 1, then twice with a low-salt buffer (5mM EDTA (pH 7.5) and 10mM Tris-HCl (pH 7.5)). Both the pellet and supernatant fractions were heat denatured for 1 minute at 100°C in Laemmli loading buffer, then subjected to SDS-PAGE (100

volts). After the electrophoretic transfer of the separated polypeptides to nitrocellulose membranes (20 volts, 16 hours, 4°C), the following antibodies were used to probe the blots: PCNA (1:500; recognizes the 36 kDa protein); DNA polymerase δ (1:3; recognizes the 125 kDa polypeptide), a generous gift from Dr. Marietta Lee; DNA polymerase α (1:250; recognizes the 180 kDa polypeptide (39)); DNA polymerase ε (1:500; recognizes the >200 kDa polypeptide); RF-C (1:500; recognizes the 140 kDa polypeptide), a generous gift from Dr. Bruce Stillman; PARP (1:750; recognizes the 116 kDa polypeptide), a generous gift from Dr. Mark Smulson; RP-A (1:500, recognizes the 34 kDa polypeptide), a generous gift from Dr. Bruce Stillman; and DNA ligase I (1:750; recognizes the 110 kDa polypeptide), a generous gift from Dr. Dana Lasko. Each blot was next incubated with the appropriate species-specific horseradish peroxidase conjugated secondary antibody and the immunodetection of the replication proteins performed with a light-enhanced chemiluminescence system. In the experiments where the Q-Sepharose peak supernatants were assayed for DNA polymerase α, DNA primase and *in vitro* DNA replication activities, co-immunoprecipitations were performed as follows. Briefly, fifty μg of the Q-Sepharose peak fraction was incubated with either 20 μg of PCNA antibody conjugated to protein A agarose beads, 20 μl of DNA polymerase α antibody (purified ascites) or 5 μg of pre-immune mouse IgG for 4 hours at 4°C on an orbital shaker. Thirty μl of protein G agarose beads, pre-coated with BSA, were next added to the reactions containing polymerase α antibody or pre-immune mouse IgG, and the incubations continued for an additional 1 hour at 4°C. The antigen-antibody complexes, bound to the protein A or G conjugated agarose beads, were pelleted by low-speed centrifugation at 3500 rpm for 5 minutes at 4°C. The protein supernatants were collected from each reaction and assayed immediately for DNA polymerase α, DNA

primase and *in vitro* SV40 DNA replication activities. The positive controls for each reaction, which were also incubated for 5 hours at 4°C, contained fifty µl of the Q-Sepharose peak fraction diluted to the appropriate level with PBS buffer.

RESULTS

DNA Ligase I and Poly(ADP-ribose)polymerase (PARP) Are Part of the Breast Cell Synthesome Core Component.

We isolated the DNA synthesome from MDA MB-468 human breast cancer cells and biopsied human breast tumor tissue according to the fractionation scheme described in our previously published procedures (12). The breast-cell-derived NE/S-3, S-4, P-4, Q-Sepharose flow-through and peak fractions (Materials and Methods) were subjected to Western blot analyses for the detection of DNA ligase I and PARP (Figures 1A and B, respectively). It was observed that both DNA ligase I and poly(ADP-ribose) polymerase (PARP) partition exclusively with the NE/S-3, P4 and Q-Sepharose peak fractions (Figures IA, IB). These proteins were not detectable in the S-4 or Q-Sepharose flow-through fractions. We have previously shown that those fractions enriched for the DNA synthesome, namely, the NE/S-3, P-4 and Q-Sepharose peak, are fully competent to support large T-antigen dependent SV40 DNA replication *in vitro* (12). It was also found that those proteins that are members of the core component of the synthesome model partition exclusively with these fractions (9-12). Therefore, our data reported here suggest that both DNA ligase I and PARP are also members of the core component of the breast cell DNA synthesome.

The Breast Cell DNA Synthesome Is a Discrete High-Molecular-Weight Complex in Native Polyacrylamide Gels.

Recent work from our laboratory indicates that the synthesome derived from HeLa cells could be isolated from native polyacrylamide gels (PAGE) as a discrete high-molecular-weight complex (Tom et al., to be published elsewhere). We report here, for the first time, that the synthesome from MDA MB-468 breast cancer cells (Figure 2, lanes 1 and 2) and human breast

tumor tissue (data not shown), also migrates as a discrete high-molecular-weight band when resolved on native polyacrylamide gels.

Protein-Protein Interactions within the Breast Cell DNA Synthesome.

To test for direct physical association between the protein members of the core component of the breast cell DNA synthesome, we performed co-immunoprecipitation studies (Materials and Methods) with the Q-Sepharose peak fraction using monoclonal antibodies directed against PCNA (mAb-10) and DNA polymerases α (SJK 132-20) and δ (mAb-11). PCNA serves as an accessory factor for DNA polymerases δ and ε, enhancing their enzymatic activities by forming a sliding clamp around duplex DNA (22,23). In accordance with its role as an auxiliary factor for DNA polymerases δ and ε, we found that PCNA antibody precipitated these two polymerases from the Q-Sepharose peak fraction in addition to the PCNA polypeptide (Figure 3A, lanes 5 and 11). Also, DNA polymerase α and RF-C were co-precipitated by the PCNA antibody (Figure 3A, lanes 7 and 9), evidence for a close interaction between PCNA and these proteins within the breast cell DNA synthesome. This result is supported by affinity chromatography studies performed with the bacteriophage T4 replication system in which a physical association between the T4 analogues of PCNA and RF-C was found (24). DNA ligase I, RP-A and PARP, on the other hand, were not co-precipitated by the PCNA antibody (Figure 3A, lanes 14, 17 and 20), suggesting that these polypeptides do not directly contact PCNA in the breast cell DNA synthesome.

Consistent with our results that PCNA interacts closely with several components of the DNA synthesome, PCNA antibody depleted the Q-Sepharose peak fraction of the discrete high-

molecular-weight band that represents the DNA synthesome on native polyacrylamide gels (Figure 2, lane 3). Moreover, PCNA antibody precipitated PCNA as well as polymerases α and δ from the human breast tumor tissue derived DNA synthesome (Figure 3A, lanes 22-24). These data obtained from human breast tissue indicate that the protein interactions detected within the DNA synthesome from MDA MB-468 breast cancer cells do not merely represent a cell line dependent phenomenon.

Further evidence for a close interaction between DNA polymerases α, δ, PCNA and RF-C within the breast cell DNA synthesome was obtained in immunoprecipitation experiments performed with DNA polymerase δ antibody (mAb-11). As shown in Figure 3B. DNA polymerase δ antibody precipitated DNA polymerase δ from the Q-Sepharose peak fraction as well as PCNA, DNA polymerase α and RF-C proteins (Figure 3B, lanes 1, 5, 7 and 9). Similar results demonstrating a close interaction between polymerases α, δ and RF-C within the RC complex from calf thymus were recently described (25). It has been proposed that synthesis of the leading and lagging strand DNA templates, mediated by DNA polymerases α and δ, respectively, is coordinated by RF-C, which acts as a molecular hinge between the two polymerases (26). Our experimental results are consistent with this theory. In contrast, we found that DNA polymerase ε, DNA ligase I, RP-A and PARP were not co-precipitated by the DNA polymerase δ antibody (Figure 3B, lanes 12, 15, 18 and 21) and probably do not directly contact DNA polymerase δ in the breast cell DNA synthesome.

As observed for the PCNA and DNA polymerase δ immunoprecipitations, DNA polymerases α and δ, PCNA and RF-C polypeptides were also precipitated by DNA polymerase α antibody (SJK 132-20) (Figure 3C, lanes 1, 3, 7 and 9). Additionally, DNA polymerase α

antibody co-precipitated RP-A, or single-stranded DNA binding protein (Figure 3C, lane 18), and PARP (Figure 3C, lane 7), which catalyzes the poly(ADP-ribosyl)ation of proteins involved in such nuclear processes as DNA replication and DNA repair (27) . Direct evidence for a physical interaction between purified RP-A and DNA polymerase α-primase proteins has been reported by other laboratories (28). Furthermore, it has been shown that PARP and DNA polymerase α purify together in 400 and 700 kDa macromolecular complexes purified from calf thymus (29) as well as interact with each other in intact 3T3-L1 cells during the initial stages of their differentiation (30).

Several lines of evidence indicate that PARP may participate in the regulation of the initiation stage of DNA synthesis. First, PARP modulates the activities of DNA polymerase α (31), topoisomerase I (32) and RP-A (27), proteins that participate in the initiation events of DNA synthesis, by catalyzing their poly(ADP-ribosyl)ation. Second, PARP can inhibit the elongation of Okazaki fragments catalyzed by DNA polymerase α during SV40 DNA replication *in vitro (27).* We have recently reported that the overall *in vitro* replication activity of the DNA synthesome is modulated by PARP *per se* and/or poly(ADP-ribosyl)ation (33) and are currently conducting experiments to determine precisely how PARP modulates the activity of the breast cell DNA synthesome.

Neither DNA polymerase ε nor DNA ligase I were co-precipitated by the DNA polymerase α antibody (Figure 3C, lanes 12, 15). These results suggest that these proteins do not directly associate with DNA polymerase α in breast cell DNA synthesome. Most importantly, the protein associations detected by the immunoprecipitations with the PCNA, DNA polymerases δ and α antibodies were specific for these antibodies. None of the components of the breast cell DNA synthesome were precipitated by a monoclonal antibody directed against the

platelet-derived growth factor (PDGF) receptor (Figure 3D, lanes 1, 5, 9, 13, 17, 20, 23, 27) or pre-immune mouse IgG (Figures 3C, lanes 3, 7, 11, 15, 19, 22, 25, 29).

DNA Polymerase α, DNA Primase and *in vitro* DNA Replication Activities of PCNA and DNA Polymerase α-Depleted Q-Sepharose Peak Fractions.

Finally, we also assayed PCNA and DNA polymerase α depleted Q-Sepharose peak fractions from MDA MB-468 breast cancer cells for DNA polymerase α, DNA primase and SV40 *in vitro* DNA replication activities. As shown in Figure 4 (lanes 2, 3, 5, 6, 8, 9), these fractions contained significantly reduced levels of DNA polymerase α, DNA primase and *in vitro* replication activities compared to control fractions. These data provide additional strong evidence for a physical association between PCNA and DNA polymerases α and δ within the breast cell DNA synthesome. Furthermore, these results indicate that DNA primase, the tightly associated subunit of DNA polymerase α, also associates with DNA polymerase δ and PCNA in the DNA synthesome. As a negative control, the Q-Sepharose peak was treated with mouse pre-immune IgG, which did not affect the ability of the breast cell DNA synthesome to support the respective enzymatic and *in vitro* replication activities (Figure 4, lanes 1, 4, 7).

DISCUSSION

In conclusion, we have demonstrated that DNA polymerases α and δ, DNA primase, PCNA and RF-C tightly associate with each other in the breast cell DNA synthesome (as suggested in our previously proposed model of the complex (12)), as each of these proteins co-precipitates with antibodies directed against polymerases α, δ and PCNA. Similar results have previously been reported for a DNA polymerization-competent, but not replication-competent, multiprotein complex purified from calf thymus (25) and in reconstituted *in vitro* replication systems (26,34). Importantly, ours is the first report ever to describe the direct physical association of DNA replication proteins within an intact replication-competent multiprotein complex isolated from human cancer cells and tumor tissue.

We have previously proposed that DNA polymerases α, δ, ε, DNA primase and RF-C constitute the core of the breast cell DNA synthesome as these polypeptides co-purify with only those fractions enriched for the DNA synthesome during the purification process (12). Based on the similar fractionation profiles for DNA ligase I and PARP, we now include these protein members of the core component of the breast cell DNA synthesome. Furthermore, PCNA may also represent a core component of the breast cancer cell synthesome as it was found to tightly associate with DNA polymerases α, δ, ε, DNA primase and RF-C. The presence of PCNA in those fractions not enriched for the DNA synthesome (9-12) may be interpreted to suggest that more than one pool of PCNA exists within the cell. In support of this claim, it has previously been reported for HeLa cells that only about 35% of the total cellular PCNA associates with replication foci during the peak of the S-phase (35).

We have constructed a new model for the organization of the proteins within the breast cell DNA synthesome based on the data presented in this report (Figure 5). Overall, our data suggest that the breast cell DNA synthesome represents an asymmetric multiprotein complex for DNA replication. According to one model for DNA replication *in vitro,* polymerase α-primase synthesizes RNA primers required for the initiation of leading strand and Okazaki fragment synthesis: whereas, DNA polymerase δ conducts leading and lagging strand DNA synthesis during the elongation phase of DNA replication (34). It is postulated that RF-C facilitates coordinated leading and lagging strand synthesis by serving as a molecular hinge between DNA polymerases α and δ. Our data support this hypothesis as we have provided direct evidence for a close association between DNA polymerases α, δ and RF-C within the breast cell DNA synthesome (Figure 5). Additionally, PCNA may participate in the coordination of leading and lagging strand synthesis by the DNA synthesome, as it was found to tightly associate with DNA polymerases α, δ and RF-C (Figure 5). Such a role for PCNA in DNA replication is supported by studies demonstrating that only early replicative lagging strand products are synthesized *in vitro* in the absence of the protein (36). Furthermore, as DNA polymerase ε represents a core component of the breast cell DNA synthesome and closely associates with PCNA in the complex, it may play a role in breast cancer cell DNA replication. Further work must be performed in order to determine the exact function of polymerase ε within the breast cell DNA synthesome. However, it has been proposed that the protein may mediate the conversion of DNA primers into Okazaki pieces (37) or act as a molecular sensor of DNA damage in eukaryotic cells (38).

We are currently conducting experiments to determine the protein members with which DNA ligase I as well as DNA topoisomerases I and II associate in the DNA synthesome. We believe that the complete characterization of the DNA synthesome will advance our understandings of the mechanisms and regulation of human breast cancer cell DNA replication. The observation that PARP modulates the *in vitro* replication activity of the DNA synthesome (33) and physically associates with DNA polymerase α in the complex (29), as demonstrated in

this report, strongly suggests that PARP may serve to regulate replication initiation events mediated by the breast cell DNA synthesome. Determining the precise mechanisms by which PARP regulates the activity of the DNA synthesome may facilitate the development of new anti-breast cancer agents capable of specifically turning off the replication activity of the complex.

ACKNOWLEDGMENTS

We would like to thank Drs. Christopher Mathews, G. Prem Veer Reddy, Ronald Berezney and Robert Freund for their critical review of the manuscript.

REFERENCES

1. Cropp. C.S. (1995) *Cancer Lett.*. **90,** 51-56.

2. Hartwell, L.H. and Kastan, M.B. (1994) *Science* **266,** 1821-1828.

3. Keshgegian, A.A. and Cnaan, A. (1995) Am. J. Clin. Pathol. **104,** 42-50.

4. Matthews, C.K. and Slabaugh, M.B. (1986) *Exp. Cell Res.* **162,** 285-295.

5. Reddy, G. and Fager, R. (1993) *Crit. Rev. Euk. Gene Exp.* **3**. 255-277.

6. Malkas, L.H., Hickey, R.J. and E.F. Baril (1990) in *The Eukarvotic Nucleus: Molecular Biochemistry and Macromolecular Assemblies,* eds. P.R. Strauss and S.H. Wilson (The Telford Press, New Jersey), pp. 45-68.

7. Tubo, R.A. and Berezney, R. (1987) *JBC* **262,** 1148-1154 .

8. Malkas, L.H., Hickey, R.J., Li, C.J., Pedersen, N. and Baril, E.F. (1990) *Biochem.* **29,** 6362-6374.

9. Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., Coll, J.M., Wills, P., Swaby, R., Wei, Y., Quan, J.Y., Lee, M.Y.W.T. and Malkas, L.H. (1995) *J. Cell. Biochem.* **59,** 91-107.

10. Wu, Y., Hickey, R.J., Lawlor, K., Wills, P., Yu, F., Ozer, H., Starr, R., Quan, J-Y., Lee M.Y.W.T. and Malkas, L.H. (1994) *J. Cell. Biochem.* **54,** 32-46.

11. Lin, S., Hickey, R.J. and Malkas, L.H. (1997) *Leuk. Res.* in press.

12. Coll, J.M., Sekowski, J.W., Hickey, R.J., Schnaper, L., Yue, W., Brodie, A.M.H., Uitto. L., Syvaoja, J.E. and Malkas, L.H. (1996) *Oncol. Res.* **8,** 435-447.

13. Tom, T., Hickey, R.J. and Malkas, L.H.(1996) *J.* Cell. Biochem. **63,** 259-267.

14. Hurwitz, J., Dean, F.B., Kwong, A.D. and Lee, S.H. (1990) *J. Biol. Chem.* **265,** 18043-18047.

15. Stillman, B. (1994) *Cell* **78,** 725-728.

16. Kelman, Z. and O'Donnell, M. (1995) *Annu. Rev. Biochem.* **64,** 171-200.

17. Sambrook, J., Fritsch, E. and Maniatis, T. (1989) in *Molecular Cloning: a laboratory manual*, 2nd edition, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp E. l-E.39.

18. Stillman, B., Gerard, R.D., Guggenheimer, R.A. and Gluzman, Y. (1985) *EMBO* **4,** 2933-2939.

19. Simanis, V. and Lane, D.P. (1985) *Virology* **144,** 88-100.

20. LaMothe, P., Baril, B., Chi, A., Lee, L. and Baril, E. (1981) *PNAS USA* **78,** 4723-4727.

21. Vishwanatha, J.K., Yamaguchi, M., DePamphilis, M.L. and Baril, E.F. (1986) *Nucl. Acids Res.* **14,** 7305-7323.

22. Prelich. G., Tan, C.K., Mikostura, M., Matthews, B., So, A.G., Downey, K.M. and Stillman, B. (1987) *Nature* **326,** 517-520.

23. Brand, S.R., Bernstein, R.M. and Mathews, M.B. (1994) *J. Immunol,.* **153**, 3070-3079.

24. Nossal, N. (1992) *FASEB* **6,** 871-878.

25. Maga, G. and Hubscher, U. *(1996) Biochem..* **35**, 5764-5777.

26. Tsurimoto, T. and Stillman, B. (1991) *J. Biol. Chem.* **266,** 1961-1968.

27. Eki, T. and Hurwitz, J. (1991) *J. Biol. Chem.* **266,** 3087-3100.

28. Dornreiter, I., Erdile, L.F., Gilbert, I.U., von Winkler, D., Kelly, T.J. and Fanning, E. (1992) *EMBO* **11**, 769-176.

29. Simbulan, C.M.G., Suzuki, M., Izuta, S., Sakurai, T., Savoysky, E., Kojima, K., Miyahara. K., Shizuta, Y. and Yoshida, S. (1995) *J. Biol. Chem.* **268,** 93-99.

30. Smulson, M., Kang, V., Ntambi, J., Rosenthal, D., Ding, R. and Simbulan, C. (1995) *J. Biol. Chem.* **270,** 119-127.

31. Yoshihara, K., Itaya, A., Tanaka, Y., Ito, K., Teraoka, H., Tsukada, K., Matsukage. A. and Kamiya, T. (1985) *Biochem. Biophys. Res. Commun.* **128,** 61-67.

32. Ferro, A.M. and Olivera, B. (1984) *J. Biol. Chem.* **259,** 547-554.

33. Simbulan-Rosenthal, C.M., Rosenthal, D.S., Hilz, H., Hickey, R.J., Malkas, L.H., Applegren, N., Wu, Y., Bers, G. and Smulson, M.E. (1996) *Biochem.* **35**, 11622-11633.

34. Waga, S. and Stillman, B. (1994) *Nature* **369,** 207-212.

35. Morris, G.F. and Mathews, M.B. (1989) *J. Biol. Chem.*, **23,** 13856-13864.

36. Prelich, G. and Stillman, B. (1988) *Cell* **53,** 117-126.

37. Nethanel, T. and Kaufmann, G. (1990) *J. Virology* **64**, 5912-5918.

38. Zlotkin, T., Kauffman, G., Jiang, Y., Lee, M.Y.W.T., Uitto, L., Syvaoja, J.E., Dornreiter, I., Fanning, E. and Nethanel, T. (1996) *EMBO* **15**, 2298-2305.

39. Tanaka, S., Hu, S.-Z., Wang, T.S.-F. and Korn, D. (1982) J. Biol. Chem. **257**, 8386-8390.

Figure Legends

Figure 1. Identification of PARP (A) and DNA ligase I (B) as core components of the breast cell DNA synthesome. Fifty μg of the NE/S-3, P-4, S-4, Q-Sepharose peak (QS peak) and Q-Sepharose flow-through (FT) protein fractions was resolved by SDS-PAGE (100 volts), then transferred to nitrocellulose membrane filters (20 volts, 16 hours, 4°C). Polyclonal antibodies directed against PARP **(A)** or DNA ligase I **(B)** were used at 1:750 dilutions to probe the membranes for either the 116 or 110 kDa proteins, respectively. Subsequently, each membrane was incubated with the appropriate species-specific horseradish peroxidase conjugated secondary antibody at a dilution of 1:5000. Immunodetection of the DNA replication proteins was performed using a light-enhanced chemiluminescence system (Amersham).

Figure 2. The breast cell DNA synthesome migrates as a discrete high molecular weight complex on native 4% polyacrylamide gels. Thirty μg of the Q-Sepharose peak (lane 1), the protein supernatant obtained after immunoprecipitation of PCNA polypeptide from 60 μl of the Q-Sepharose peak by PCNA antibody (mAb-10; 2.5 μg) (lane 3), and a positive control for this reaction containing 60 μl of the Q-Sepharose peak diluted to the appropriate level with PBS buffer (lane 2), were resolved by native polyacrylamide gel electrophoresis (90 volts, 4°C), then transferred to nitrocellulose membrane filters (15 volts, 16 hours, 4°C). A monoclonal directed against DNA polymerase α antibody (SJK 132-20) was used at a 1:400 dilution to probe the membrane for the high molecular weight band that represents the DNA synthesome. In order to demonstrate that PCNA antibody had completely precipitated PCNA polypeptide from the Q-Sepharose peak in these experiments, we subjected both the pellet (lane 5) and supernatant (lane 6) fractions obtained in a parallel immunoprecipitation experiment to SDS-PAGE. After electrophoretic transfer of the resolved polypeptides to nitrocellulose membrane filters, PCNA

antibody was used at a 1:500 dilution to probe the membrane for the 36 kDa protein. Lane 4 shows PCNA contained in 50 μg of the Q-Sepharose peak (positive control).

Figure 3. Co-immunoprecipitation of several of the protein components of the DNA synthesome by monoclonal antibodies directed against PCNA (mAb-10) (A), DNA polymerase δ (mAb-11) (B) and DNA polymerase α (SJK 132-20) (C). Negative control co-immunoprecipitations **(D)** were performed using either a purified monoclonal antibody recognizing the PDGF receptor or pre-immune mouse IgG. For each panel, positive control lanes contained 50 μg of the Q-Sepharose peak probed with the respective antibodies. The target proteins are marked by a bullet and the antibody heavy and light chains (IgG) are marked by arrows. IP represents an abbreviation for immunoprecipitation.

Figure 4. DNA polymerase α, DNA primase and *in vitro* SV40 DNA replication activities of the Q-Sepharose peak fraction treated with monoclonal PCNA (mAb-10) and DNA polymerase α (SJK 132-20) antibodies. The Q-Sepharose peak fraction was incubated with PCNA antibody conjugated to protein A agarose beads (lanes 2, 5 and 8), DNA polymerase α antibody (purified ascites) (lanes 3, 6 and 9) or pre-immune mouse IgG (lanes 1, 4 and 7). After the antigen-antibody-protein A/G agarose complexes were pelleted by low-speed centrifugation, the protein supernatants were collected and assayed immediately for DNA polymerase α, DNA primase and *in vitro* DNA replication activities. Diagonally striped bars represent DNA polymerase α activity. Checked bars represent DNA primase activity, while vertical striped bars represent *in vitro* DNA replication activity. Each value for percent control activity (cpm) is an average of 2-5 separate experiments. Lines represent deviations from the average. Lane 10

represents the *in vitro* DNA replication activity contained in both PCNA and polymerase α depleted Q-Sepharose peak fractions. The typical control values for DNA polymerase α, DNA primase and *in vitro* DNA replication activities are 5,253 cpm, 8,083 cpm and 103,336 cpm, respectively.

Figure 5. A model for the human breast cell DNA synthesome.

A.

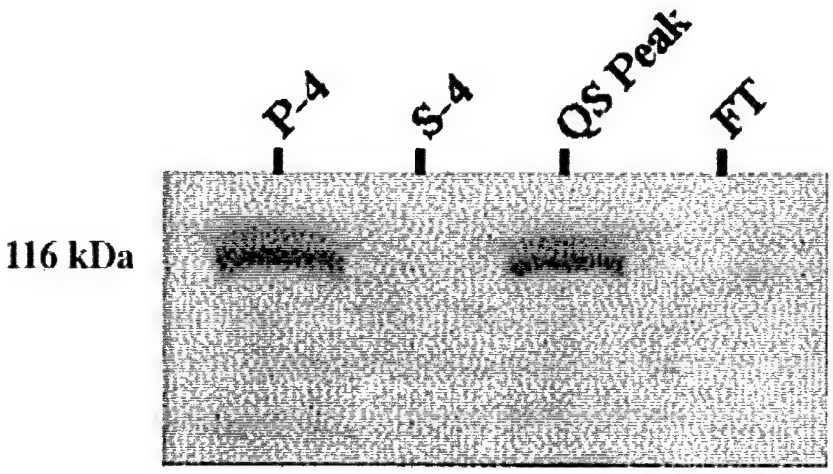


Figure 1A

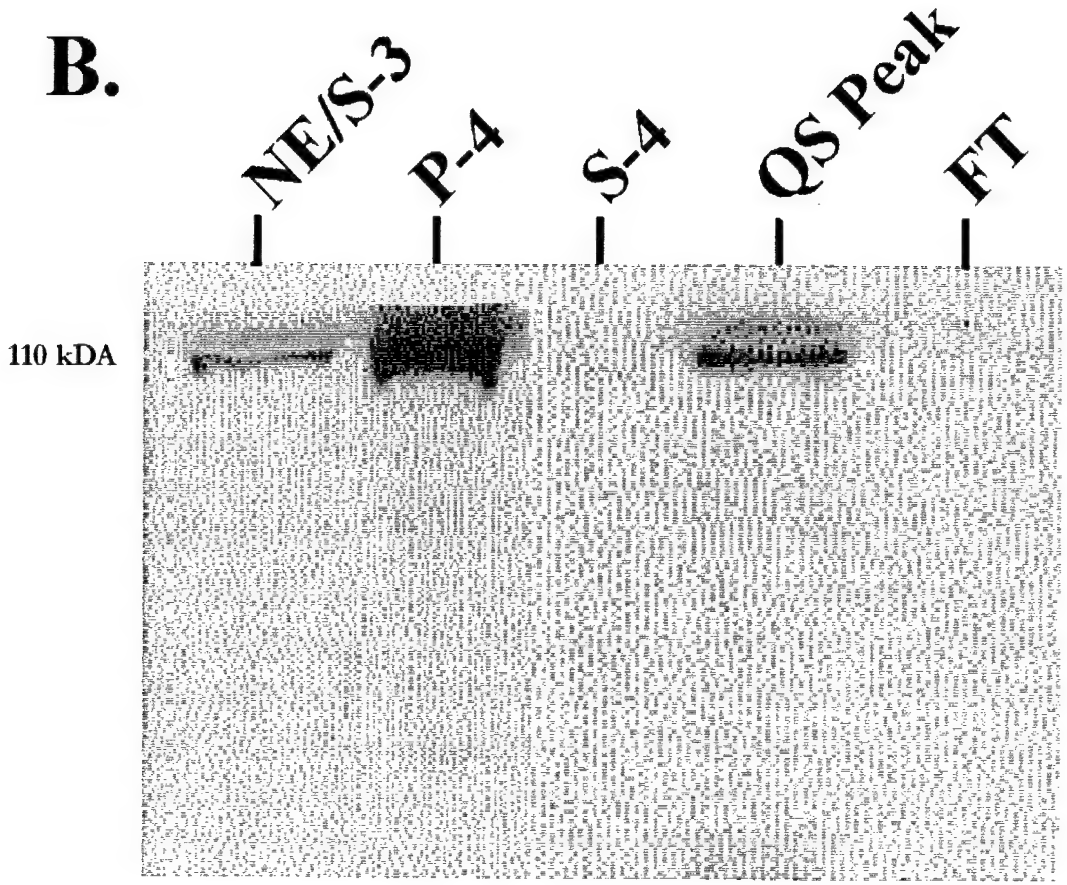


Figure 1B

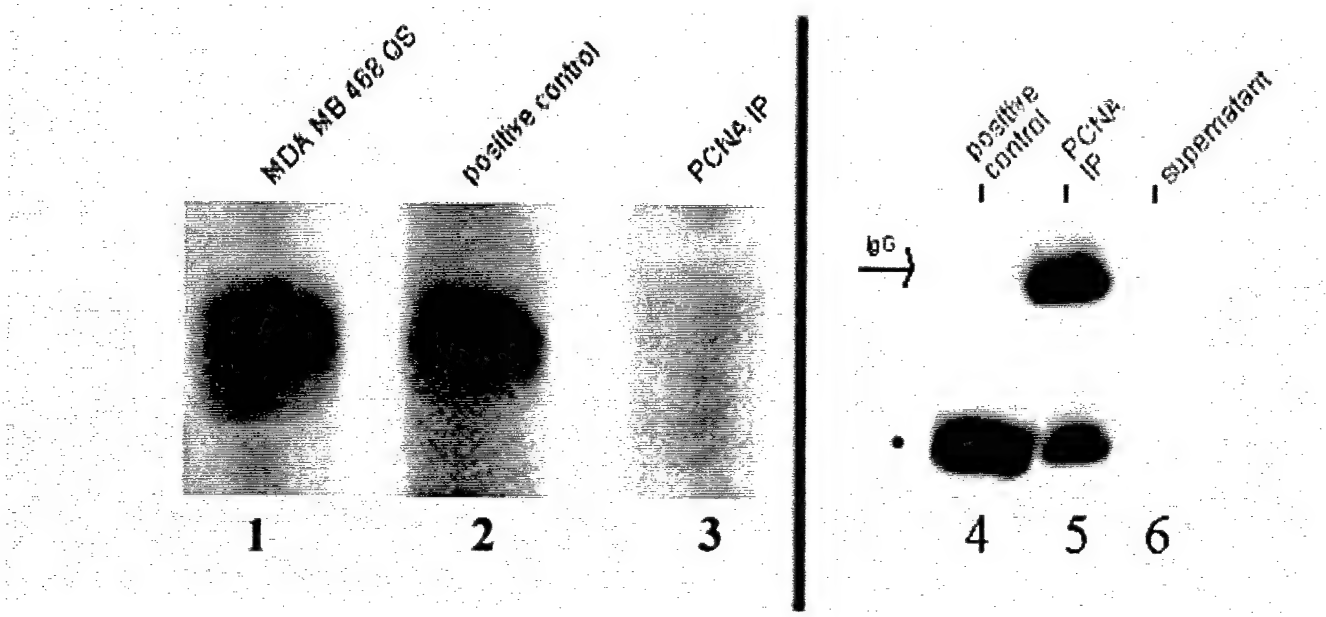

MDA MB 468 GS
positive control
PCNA IP
1
2
3
bG
positive control
PCNA IP
supernatant
4
5
6

Figure 2

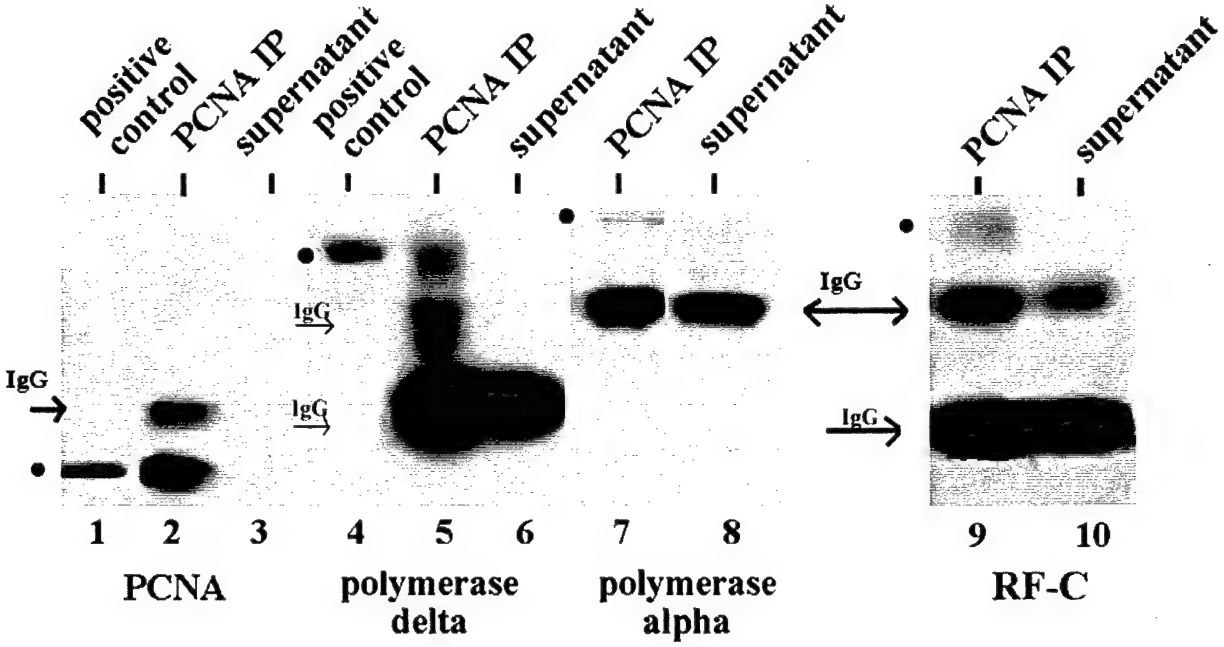

positive control
PCNA IP
supernatant
positive control
PCNA IP
supernatant
PCNA IP
supernatant
PCNA IP
supernatant
IgG
IgG
IgG
IgG
1
2
3
4
5
6
7
8
9
10
PCNA
polymerase delta
polymerase alpha
RF-C

Figure 3A(1)

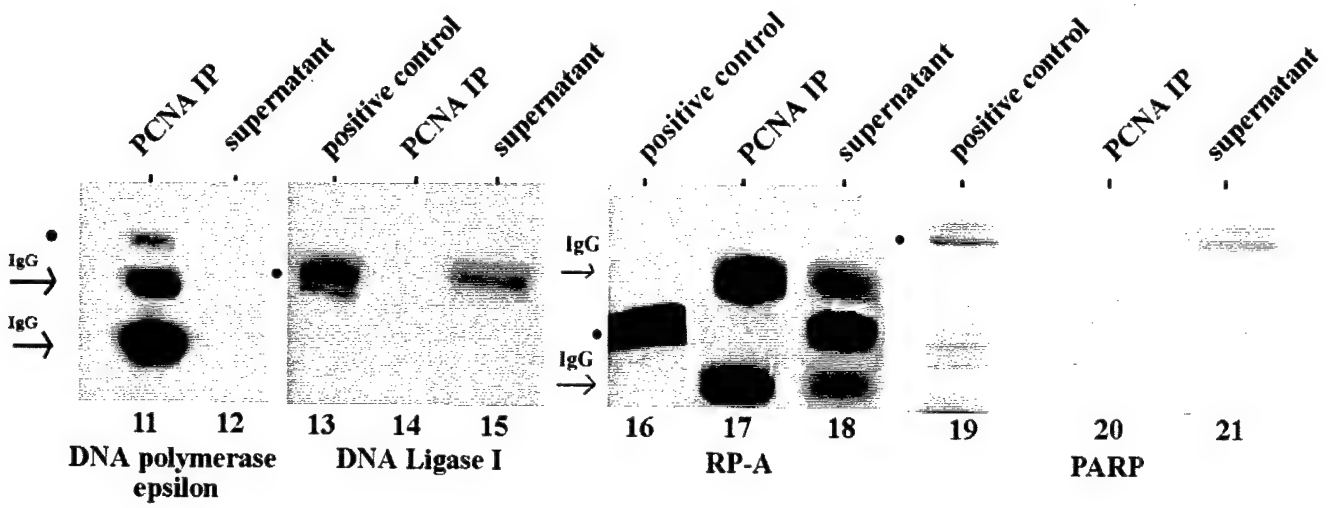

PCNA IP
supernatant
positive control
PCNA IP
supernatant
positive control
PCNA IP
supernatant
positive control
PCNA IP
supernatant
IgG
IgG
IgG
IgG
11
12
13
14
15
16
17
18
19
20
21
DNA polymerase epsilon
DNA Ligase I
RP-A
PARP

Figure 3A(2)

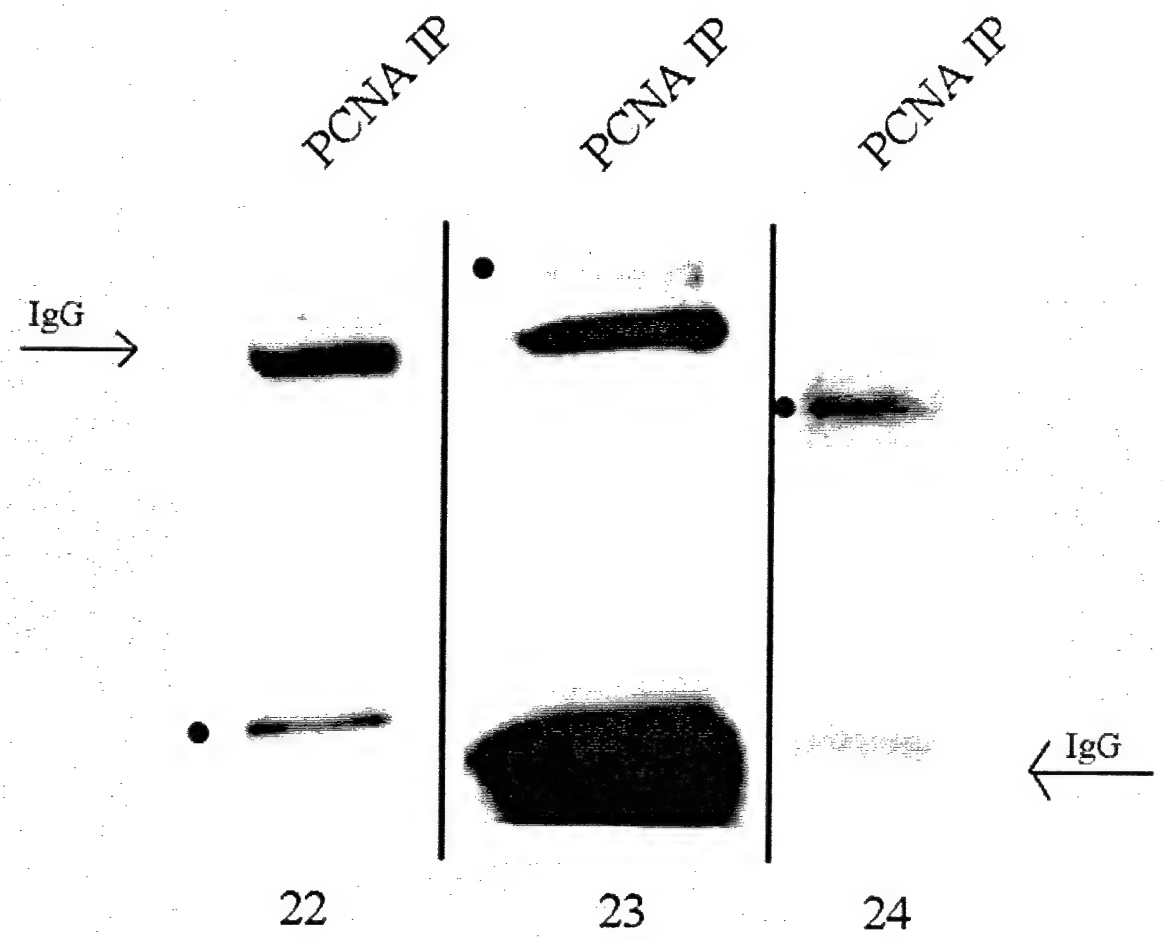


Figure 3A(3)

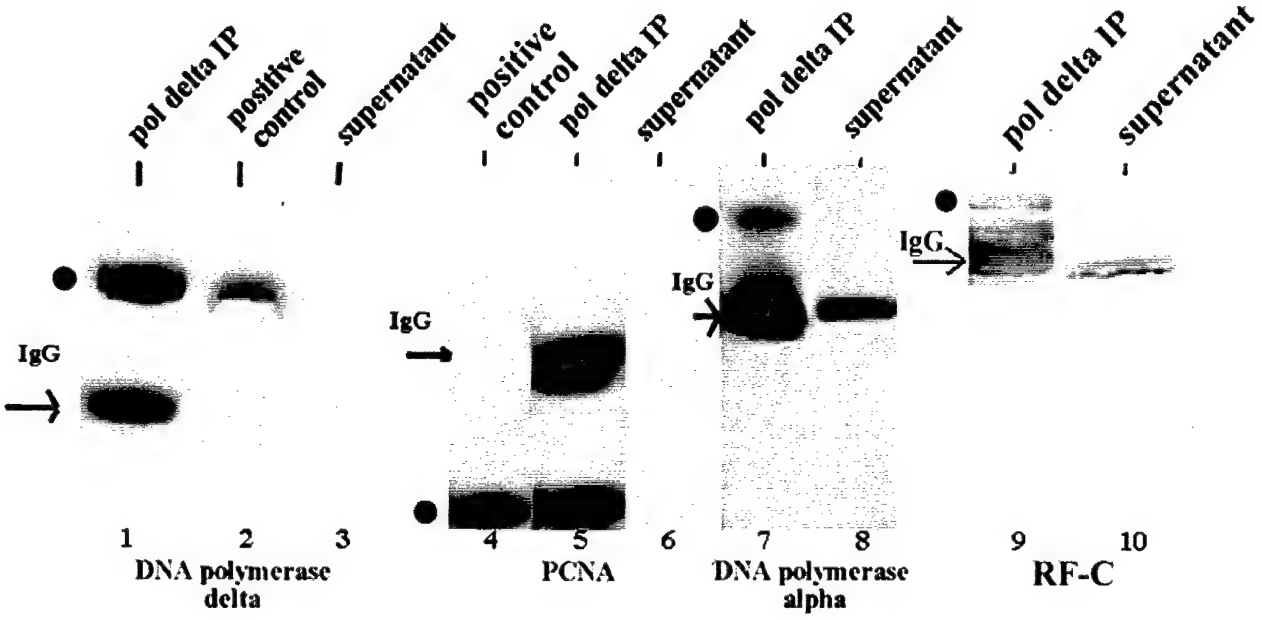

pol delta IP
positive control
supernatant
positive control
pol delta IP
supernatant
pol delta IP
supernatant
pol delta IP
supernatant
IgG
IgG
IgG
IgG
1
2
3
4
5
6
7
8
9
10
DNA polymerase delta
PCNA
DNA polymerase alpha
RF-C

Figure 3B(1)

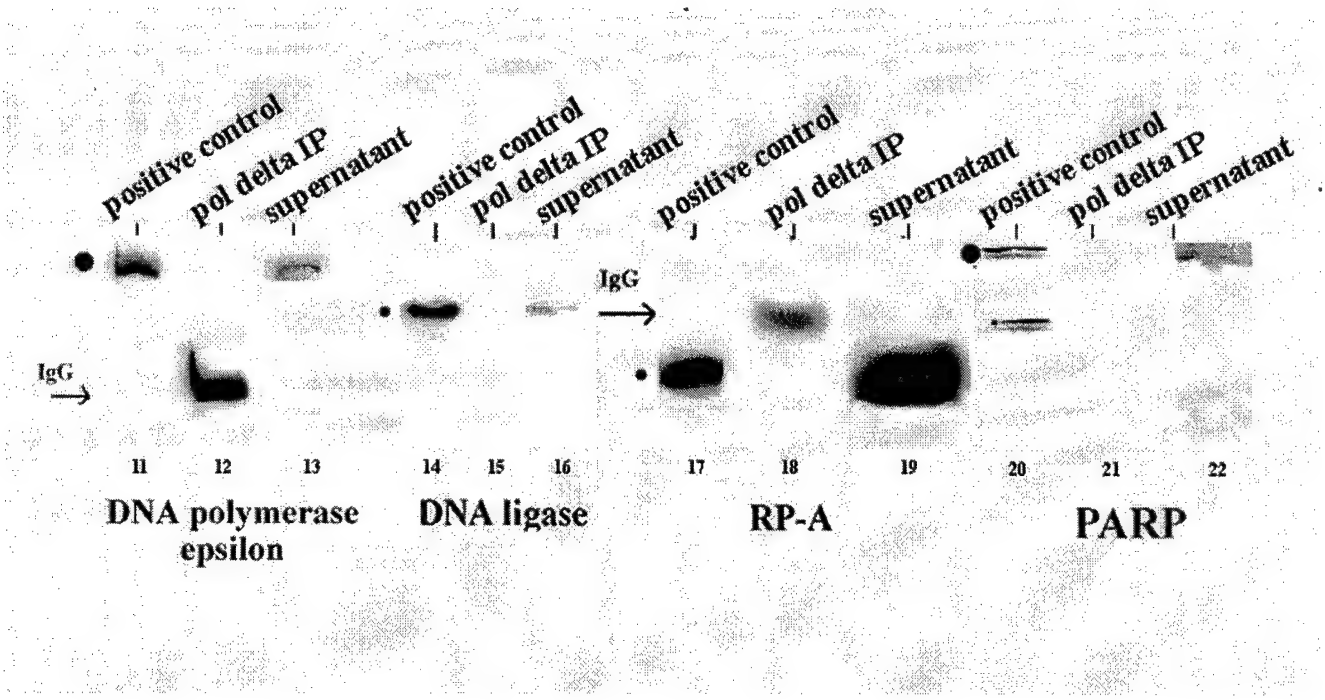

positive control
pol delta IP
supernatant
positive control
pol delta IP
supernatant
positive control
pol delta IP
supernatant
positive control
pol delta IP
supernatant
IgG
IgG
11
12
13
14
15
16
17
18
19
20
21
22
DNA polymerase epsilon
DNA ligase
RP-A
PARP

Figure 3B(2)

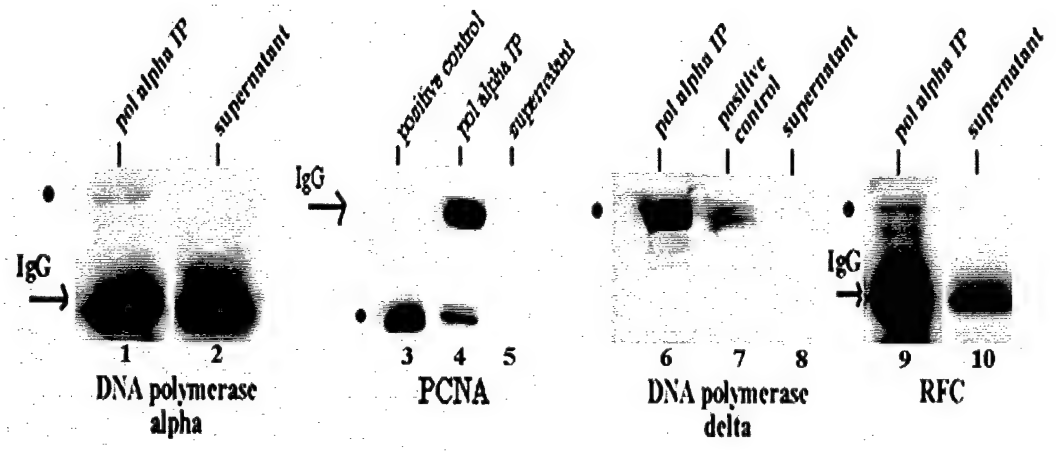

pol alpha IP
supernatant
IgG
1
2
DNA polymerase alpha
positive control
pol alpha IP
supernatant
IgG
3
4
5
PCNA
pol alpha IP
positive control
supernatant
6
7
8
DNA polymerase delta
pol alpha IP
supernatant
IgG
9
10
RFC

Figure 3C(1)

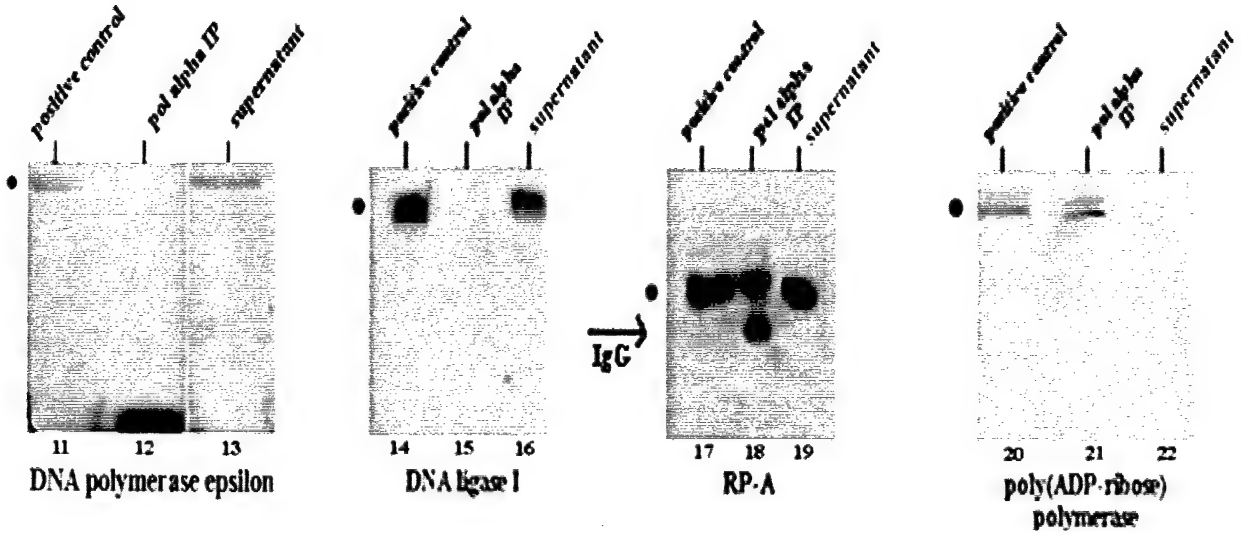

positive control
pol alpha IP
supernatant
11
12
13
DNA polymerase epsilon
positive control
pol alpha IP
supernatant
14
15
16
DNA ligase I
IgG
positive control
pol alpha IP
supernatant
17
18
19
RP-A
positive control
pol alpha IP
supernatant
20
21
22
poly(ADP-ribose) polymerase

Figure 3C(2)

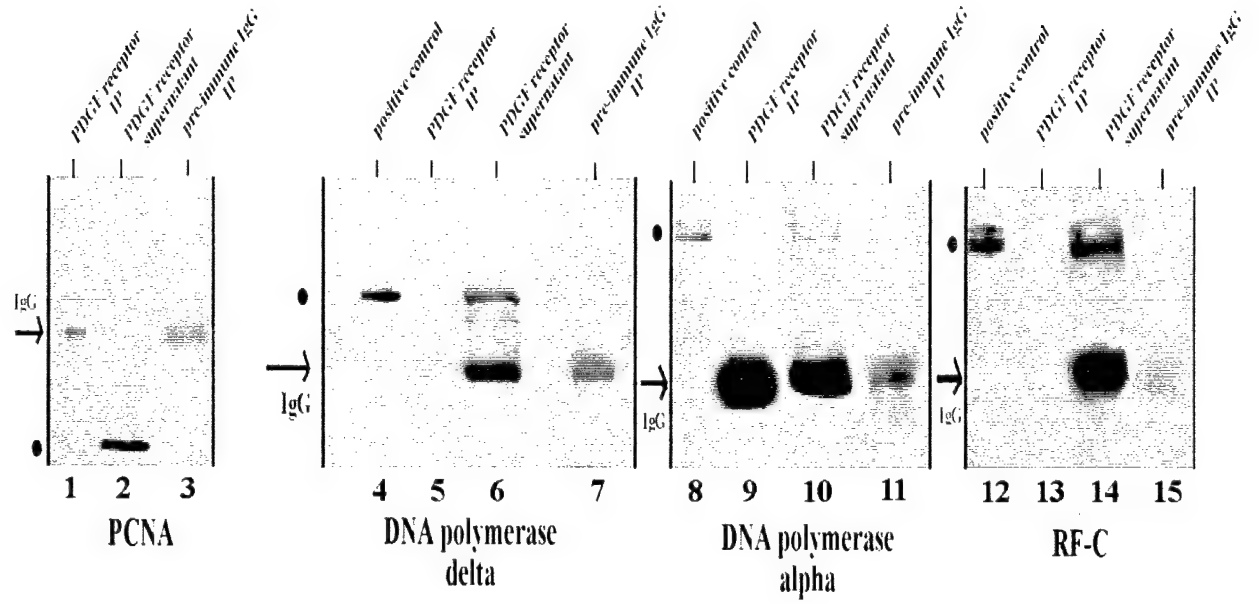

PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
positive control
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
positive control
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
positive control
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
IgG
IgG
IgG
IgG
1 2 3
PCNA
4 5 6 7
DNA polymerase delta
8 9 10 11
DNA polymerase alpha
12 13 14 15
RF-C

Figure 3D(1)

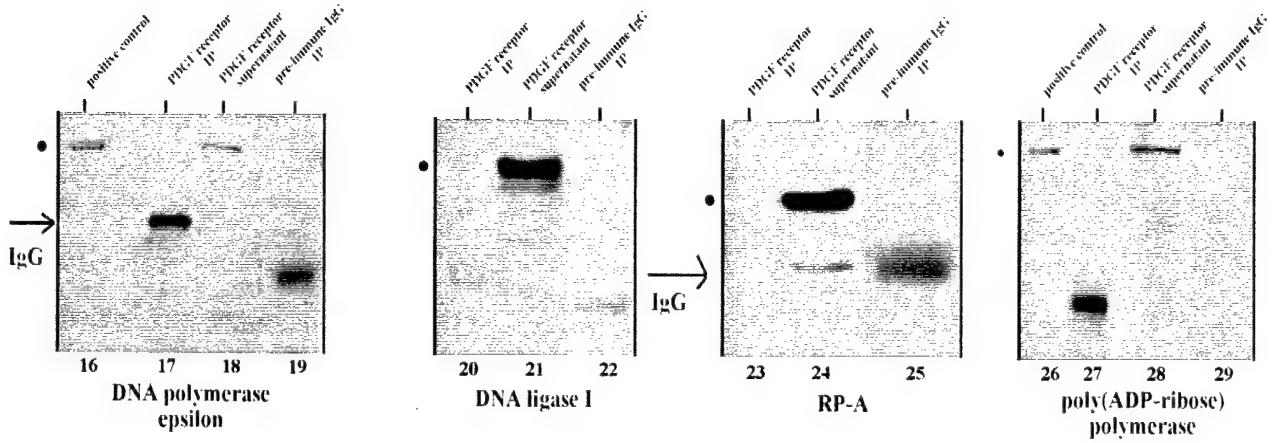
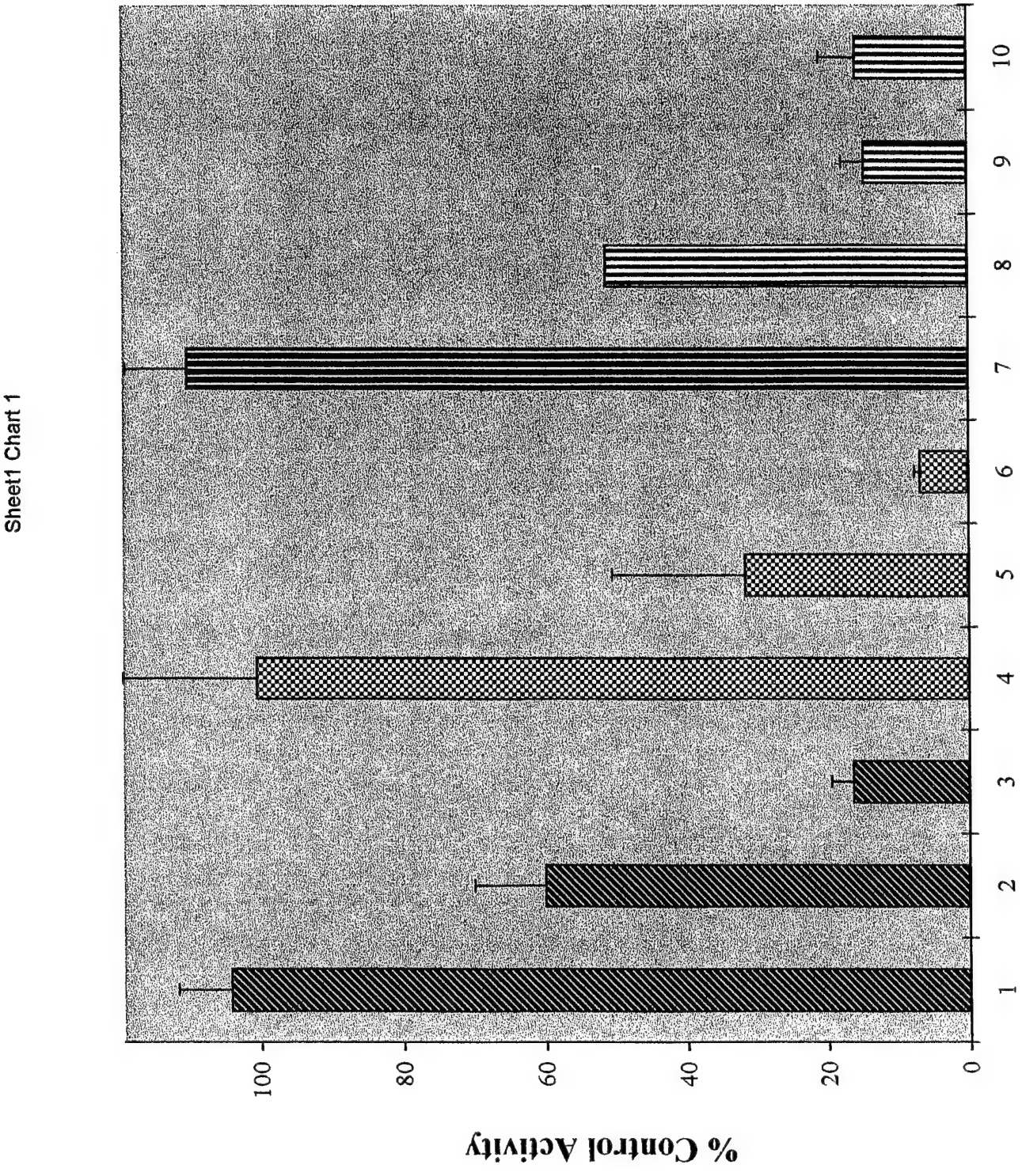

positive control
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
IgG
16
17
18
19
DNA polymerase epsilon
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
20
21
22
DNA ligase I
IgG
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
23
24
25
RP-A
positive control
PDGF receptor IP
PDGF receptor supernatant
pre-immune IgG IP
26
27
28
29
poly(ADP-ribose) polymerase

Figure 3D(2)

Figure 4


Sheet1 Chart 1
% Control Activity
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10

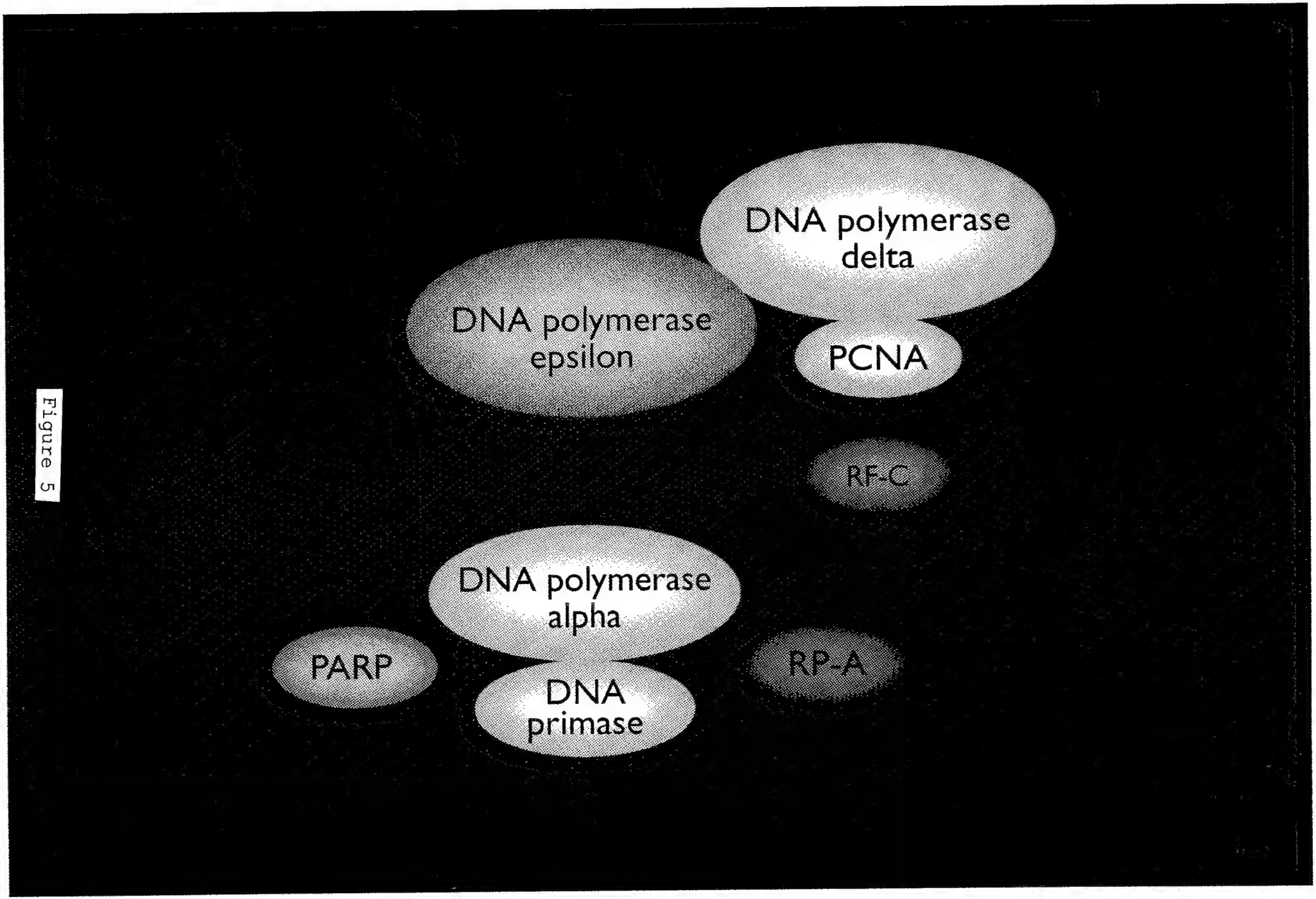

DNA polymerase delta
DNA polymerase epsilon
PCNA
RF-C
DNA polymerase alpha
PARP
RP-A
DNA primase

Figure 5

The Breast Cell DNA Synthesome: A Novel Model for Examining the Mechanisms of Action of Irenotecan and Etoposide

Jennifer M. Coll [(1)], Erica A. Cronkey [(1)], Robert J. Hickey [(2-5)], Lauren Schnaper [(6,7)] and Linda H. Malkas [(1, 3-5) *]

[1]Department of Pharmacology and Experimental Therapeutics, [3]Program in Molecular and Cellular Biology, [4]Program in Oncology, [5]Program in Toxicology, [6]Department of Surgery, University of Maryland School of Medicine, Baltimore, MD 21201; [2]Department of Oral Craniofacial Biological Sciences, University of Maryland School of Dentistry, Baltimore, MD 21201; [7]Breast Evaluation and Treatment Center, Greater Baltimore Medical Center, Baltimore, MD 21240.

Running Title: A Novel Model for Examining the Mechanisms of Action of CPT-11 and VP-16

Key Words: DNA replication; anticancer agent; multiprotein complex; breast cancer

Title Footnote:

***Author: Linda H. Malkas,** author to whom correspondence should be addressed

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics
(3) Program in Molecular and Cellular Biology
(4) Program in Oncology
(5) Program in Toxicology
685 W. Baltimore St.
Baltimore, MD 21201

Tel: (410) 706-2313 or 1798
Fax: (410) 706-0337

Author: Jennifer M. Coll

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics

Author: Erica A. Cronkey

Address: University of Maryland School of Medicine
(1) Department of Pharmacology and Experimental Therapeutics

Author: Robert J. Hickey

Address: University of Maryland School of Dentistry
(2) Department of Oral Craniofacial Biological Sciences
(3) Program in Molecular and Cellular Biology
(4) Program in Oncology
(5) Program in Toxicology

Author: Lauren Schnaper

Address: Greater Baltimore Medical Center
(7) Breast Evaluation and Treatment Center
(6) Department of Surgery

ABSTRACT

We have previously described the isolation and characterization of an intact multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer (MDA MB-468) cells and biopsied human breast tumor tissue. We have shown that the proteins and enzymes constituting the breast cell DNA synthesome include: DNA polymerase α, proliferating cell nuclear antigen (PCNA), DNA polymerase δ, DNA primase, replication factor C (RF-C), replication protein A (RP-A), DNA ligase I, DNA polymerase ε and DNA topoisomerases I and II. In the presence of the viral large T-antigen, the breast cell DNA synthesome is fully capable of supporting the replication of simian virus 40 (SV40) origin-containing DNA *in vitro*. Moreover, as shown in this report, the daughter DNA molecules produced by the breast cell DNA synthesome consist of monomeric, closed circular form I DNA's as well as topological and replicative intermediates that are resistant to digestion by *Dpn*I; which is consistent with the criteria for semi-conservative replication. As the DNA synthesome represents the intact breast cell's DNA replication machinery, it may serve as a novel model for examining the mechanisms of action of anti-breast cancer agents that target the DNA synthetic process. In this report, we present for the first time data that indicate the breast cell DNA synthesome serves as a powerful model for studying the actions of irenotecan (CPT-11) and etoposide (VP-16)--two anti-cancer agents that trap nuclear topoisomerases I and II, respectively, in ternary cleavable complexes. We found a close correlation between the IC_{50} values for the inhibition of intact cell and DNA synthesome-mediated *in vitro* DNA replication by CPT-11 (SN-38) (2.0 μM and 0.2 μM, respectively) and VP-16 (2 μM and 0.5 μM, respectively). Additionally, we found that similar concentrations of SN-38 and VP-16, 0.5 μM each, inhibited topoisomerase I and II enzymatic activities by 50% as well as produced significant levels of cleavable complexes. Consistent with these findings, alkaline agarose gel electrophoretic analysis of the DNA products synthesized *in vitro* indicate that SN-38 and VP-16 (0.2 μM and 0.5 μM, respectively) inhibit the elongation of nascent DNA molecules by the DNA synthesome; the latter result suggesting that topoisomerase II functions during DNA chain

elongation. Ultimately, utilization of the breast cell DNA synthesome as a model for studying the mechanisms of action of CPT-11 and VP-16 may provide insight into the lethal events that occur beyond drug-stabilized cleavable complex formation as well as aid the development of improved analogues of these agents.

INTRODUCTION

Eukaryotic DNA topoisomerases I and II are nuclear enzymes which play critical roles in DNA replication and chromosomal segregation by catalyzing the topological isomerization of DNA via breakage-reunion reactions [1]. Topoisomerase I, a 100 kDa monomeric protein, relaxes both positively and negatively supercoiled DNA in an energy-independent reaction by introducing a transient single-strand nick into the DNA phosphodiester backbone [2]. This action enables the enzyme to facilitate origin unwinding during the initiation stage of DNA synthesis [3] and relax positive DNA supercoils as they accumulate ahead of the replication fork during DNA chain elongation [4, 5]. Different from topoisomerase I, topoisomerase II, a 180 kDa homodimeric polypeptide, which introduces transient double-strand breaks into the DNA phosphodiester backbone in an ATP-dependent reaction [6]. This action also facilitates the relaxation of positively supercoiled DNA during chain elongation and permits the passage of a DNA duplex, necessary for the segregation of newly synthesized daughter DNA molecules [4, 5].

In addition to playing critical roles in DNA replication and several other DNA metabolic processes, topoisomerases I and II serve as the primary targets of a number of clinically relevant antineoplastic agents [7, 8]. Two such agents that have demonstrated efficacy against breast cancer in clinical trials and are utilized in multi-drug chemotherapy regimens against the disease are irenotecan (CPT-11) and etoposide (VP-16), respectively. CPT-11 and VP-16 trap topoisomerases I and II, respectively, in ternary (drug-enzyme-DNA) cleavable complexes, in which the enzymes remain covalently bound to DNA [7-9]. In this state, neither topoisomerase I nor II can perform its DNA nicking-resealing function. It has been demonstrated that the accumulation of drug-stabilized cleavable complexes correlates with irreparable DNA damage and cytotoxicity [10, 11]. However, the precise mechanisms by which cleavable complex formation culminates in cell death are currently unknown.

Numerous reports have demonstrated that simian virus 40 (SV40) DNA replication *in vitro* represents a potential model for studying the mechanisms of action of anticancer agents that target mammalian cell DNA synthesis [12, 13]. We have previously described the isolation and characterization of an intact multiprotein complex for DNA synthesis, designated the DNA synthesome, from human cervical (HeLa) and murine mammary (FM3A) carcinoma cells [14-17] and most recently from human breast cancer (MDA MB-468) cells as well as from biopsied human breast tumor tissue [18]. In the presence of the viral large T-antigen and SV40 origin sequences, the human cell DNA synthesome fully supports semi-conservative SV40 DNA replication *in vitro.* The replication activity of the DNA synthesome is also completely dependent on Mg^{2+}, ribonucleoside triphosphates, deoxyribonucleoside triphosphates and a renewable source of ATP provided by phosphocreatine kinase and phosphocreatine [19]. These requirements for DNA synthesome mediated *in vitro* DNA replication are comparable to those observed for cells permissive for SV40 infection [20], strongly suggesting that the DNA synthesome represents the intact cell's DNA replication machinery. We have demonstrated that the integrity of the human cell DNA synthesome is maintained after its treatment with salt, detergent, RNase, DNase, anion-exchange chromatography, sucrose or glycerol gradient sedimentation and electrophoresis through native polyacrylamide gels [17, 18, 21]. These results suggest that the association of the proteins with one another is independent of non-specific interactions with other cellular macromolecules. Furthermore, we have shown that the following proteins and enzymes constitute the human cell DNA synthesome: DNA polymerase α-primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), replication protein A (RP-A), DNA helicases II and IV, DNA ligase I and fully functional DNA topoisomerases I and II.

In this report, we describe experiments that establish that the DNA synthesome, isolated from MDA MB-468 human breast cancer cells, serves as a novel model for examining the mechanisms of action of CPT-11 and VP-16. Ultimately, an improved understanding of the

modes of action of CPT-11 and VP-16 may aid the development of improved analogues of these agents for breast cancer treatment.

MATERIALS AND METHODS

Materials. CPT and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). CPT-11 was kindly supplied by Dr. John Wilkes of Pharmacia/UpJohn (Kalamazoo, MI). All drugs were dissolved in dimethyl sulfoxide at stock concentrations of 5.0 mM and stored at -20°C.

Purified DNA topoisomerases I (2 units/μl) and II (2 units/μl) were purchased from TopoGen, Inc. (Columbus, Ohio). One unit of topoisomerase I relaxes 250 ng supercoiled DNA in 30 minutes at 37°C; while, one unit of topoisomerase II decatenates 200 ng *Crithidia fasciculata* K DNA networks in 30 minutes at 37°C.

α[^{32}P]dCTP and α[^{32}P]dATP (3000 Ci/mmol; 370 MBq/ml; 10 mCi/ml) as well as [^{3}H]-thymidine (90 Ci/mmol; 37 MBq/ml; 2.5 mCi/ml) were obtained from DuPont New England Nuclear (Boston, MA).

Methods

Cell Culture. Suspension cultures of MDA MB-468 human breast carcinoma cells were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated new-born calf serum and fetal bovine serum. Exponentially growing cells ($5x10^5$ cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS): 20 mM Na_2HP0_4, 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 minutes, 4°C), and the cell pellets stored at -80°C until fractionation.

Isolation and purification of the DNA synthesome from MDA MB-468 breast cancer cells. MDA MB-468 breast cancer cells (15 g) were homogenized and the breast cell DNA synthesome was purified as described in our previously published procedures [18]. The protein fraction designated the Q-Sepharose peak (0.9 μg/μl), which contains the replication-competent DNA synthesome, was used in the experiments described in this report.

Measurement of intact MDA MB-468 cell DNA synthesis. Exponentially growing MDA MB-468 breast cancer cells ($5x10^4$ per dish) were incubated at 37°C with increasing concentrations of CPT-11, CPT or VP-16 in the presence of [^{3}H]-thymidine. After a one hour incubation, cells were lysed and the amount of radiolabel incorporated into DNA was determined by the isolation and counting of acid-insoluble material [22].

***In vitro* SV40 DNA replication assay.** Assay reaction mixtures (12.5 µl) contained 80 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 1 mM DTT; 5-10 µg of the Q-Sepharose peak; 0.5-1.0 µg of purified SV40 large T-antigen; 25 ng of plasmid pSVO$^+$ containing an insert of SV40 replication-origin DNA sequences [23]; 100 µM each dTTP, dATP, dGTP; 200 µM each rCTP, rGTP, rUTP; 4mM ATP; 50 µM α[^{32}P]-dCTP; 40 mM creatine phosphate; 1µg creatine phosphokinase. The standard reaction, conducted in the absence or presence of increasing concentrations of SN-38, CPT or VP-16, was incubated for 2 hr at 37°C. Replication assay products were processed using DE81 filter binding to determine the amount of radiolabel incorporated into acid insoluble material [22].One unit of SV40 replication activity is equivalent to the incorporation of 1 pmol of dNMP into newly synthesized DNA per 1h under the standard assay conditions. For neutral and alkaline agarose gel electrophoretic analyses, DNA replication assays (25 µl) were performed for 1 hour; the DNA products were processed as described in a later section of these Materials and Methods.

DNA topoisomerase I assay. DNA synthesome-associated topoisomerase I activity was measured by incubating 150 ng of supercoiled pSVO$^+$ DNA with 8 µg of the Q-Sepharose peak in a buffer containing: 10 mM Tris-HCl (pH 7.9); 1 mM EDTA; 0.15 M NaCl; 0.1% BSA; 0.1

mM spermidine and 5% glycerol. In order to examine the effects of SN-38 and CPT on DNA synthesome-associated topoisomerase I activity, reactions were also performed in the presence of increasing concentrations of these agents. Reactions were incubated for 15 minutes at 37°C. Each reaction (20 μl) was stopped by adding 2 μl of 10% SDS and the DNA products resolved on a 1.0 % agarose gel containing TAE buffer (40 mM Tris acetate, 2 mM EDTA). After ethidium bromide (1 μg/ml) staining of the gels [22], topoisomers were visualized with an ultraviolet light source.

DNA topoisomerase II assay. DNA synthesome-associated topoisomerase II activity was measured by incubating 260 ng of *Crithidia fasciculata* K DNA networks with 8 μg of the Q-Sepharose peak in a buffer containing: 50 mM Tris-HCl (pH 8.0); 120 mM KCl; 10 mM $MgCl_2$; 0.5 mM ATP; 0.5 mM DTT; 30 μg/ml BSA. In order to examine the effects of VP-16 on DNA synthesome-associated topoisomerase II activity, reactions were also performed in the presence of increasing concentrations of the agent. Reactions were incubated for 30 minutes at 37°C. Each reaction (20 μl) was stopped by adding EDTA to a final concentration of 25 mM; protein was then digested with 50 ng/μl of proteinase K for an additional 15 minutes at 37°C. After the addition of gel loading buffer and extraction with chloroform/isoamyl alcohol, the DNA products were resolved at 80 volts on a 1% agarose gel containing TBE buffer (50 mM Tris-borate; 1 mM EDTA) and ethidium bromide (0.5 μg/ml). DNA was visualized with an ultraviolet light source.

Quantitation of the amount of topoisomerases I and II present in the Q-Sepharose peak. One unit of DNA topoisomerase I activity is sufficient to fully convert 250 ng of supercoiled, form I DNA into open-circle, form II DNA in 30 minutes at 37°C. The minimal amount of DNA synthesome-associated topoisomerase I necessary to fully convert 250 ng of supercoiled, form I DNA to open-circular, form II DNA was determined by performing topoisomerase I assays with

dilutions of the Q-Sepharose peak. Using this approach, we estimated that 8 µl (8 µg) of the Q-Sepharose peak contains one unit of DNA topoisomerase I activity. The amount of DNA topoisomerase II present in the Q-Sepharose peak was determined by the method described above, where one unit of topoisomerase II activity is sufficient to decatenate 200 ng of K DNA networks in 30 minutes at 37°C. We estimated that 8 µl of the Q-Sepharose peak contains one unit of DNA topoisomerase II activity.

3' end-labeling of pSVO$^+$ plasmid DNA. 10 µg pSVO$^+$ plasmid DNA was incubated with 20 units EcoRI restriction endonuclease (New England BioLabs, Boston, MA) in 1X EcoRI buffer overnight at 37°C. The cut DNA (10 µg) was incubated with 5 units of Klenow fragment (Stratagene, LaJolla, CA) in the presence of 5 mM each dATP and TTP and 80 µCi $\alpha[^{32}P]$dATP for 1 hour at 37°C. Reactions were stopped by adding EDTA to a final concentration of 25 mM; the reactions were then diluted to 70 µl with STE buffer containing: 0.1 M NaCl; Tris-HCl (pH 8.0); and 1 mM EDTA. Unincorporated deoxynucleoside triphosphates were removed by chromatography through a P60 gel filtration column [22].

SDS precipitation of DNA synthesome-associated topoisomerase I and II cleavable complexes. Cleavage of DNA by topoisomerases I and II was performed as follows. Briefly, 10 µg of the Q-Sepharose peak was incubated with 220 ng of 3' end-labeled pSVO$^+$ DNA and increasing concentrations of either SN-38, CPT or VP-16 in topoisomerase I or II reaction buffer. Incubations were performed for 30 minutes at 37°C. Reactions (30 µl) were stopped by the addition of 0.1 volume of 10% SDS and 250 µl of buffer A1 (TopoGen Inc.) containing: 10 mM Tris-HCl (pH 7.5); 20 µg/ml BSA; 20 µg/ml calf thymus DNA and 1% SDS. Double-stranded topoisomerase-DNA cleavable complexes were precipitated by KCl (added to a final concentration of 0.2 M) followed by incubation on ice for 15 minutes. The precipitated

topoisomerase-DNA cleavable complexes were isolated by filtration onto glass fiber filters, and processed according to the manufacturer's guidelines (TopoGen Inc.).

Neutral and alkaline gel electrophoretic analyses of the DNA products synthesized by the DNA synthesome. For both neutral and alkaline agarose gel electrophoretic analyses of the DNA products, the DNA replication reactions (25 μl) were terminated by the addition of EDTA to 25 mM, SDS to 1% and 50 μg yeast tRNA as a carrier. Next, the protein contained in the DNA replication reactions was digested with 50 ng/μl proteinase K for 1 hour at 37°C. The DNA products were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (24:25:1), followed by chloroform/isoamyl alcohol (24:1). The DNA products were precipitated by 2.0 M ammonium acetate and 100% ethanol, then washed three times with 70% ethanol. For neutral agarose gel electrophoresis, the DNA pellets were resuspended in 20 μl TE buffer: 10 mM Tris (pH 7.4) and 0.1 mM EDTA (pH 8.0). After the addition of gel loading buffer, the DNA products were resolved overnight at 30 volts on a 1.2% agarose gel containing TBE buffer. For analysis of the denatured DNA products, DNA pellets were resuspended in 15 μl of a buffer containing 50 mN NaOH and 1 mM EDTA. After the addition of alkaline gel loading buffer, the DNA was resolved overnight at 30 volts on a 1% alkaline agarose gel containing 50 mN NaOH and 1 mM EDTA. Following electrophoresis, the resolved DNA products were fixed with 7% trichloroacetic acid. Both the neutral and alkaline agarose gels were dried and subjected to autoradiography in order to visualize the replication products.

RESULTS

Inhibition of intact cell and DNA-synthesome mediated in vitro DNA replication by CPT-11 (SN-38) and VP-16

In order to demonstrate that CPT-11, as well as its parent compound camptothecin (CPT), and VP-16 are potent inhibitors of cellular DNA replication, we measured the level of DNA synthesis in intact MDA MB-468 breast cancer cells exposed to increasing concentrations of these agents. Briefly, exponentially growing MDA MB-468 breast cancer cells were exposed for one hour to logarithmically increasing concentrations of either CPT-11, CPT or VP-16 in the presence of [^{3}H]-thymidine. Afterward, cellular DNA replication activity was determined by quantitating the amount of [^{3}H]-TMP incorporated into acid-insoluble material (Materials and Methods). As shown in Figure 1, the exposure of breast cancer cells to increasing concentrations of either CPT-11, CPT or VP-16 resulted in a dose-dependent decrease in [^{3}H]-TMP incorporation into cellular DNA. DNA replication activity was inhibited approximately 50% by 2.0 μM CPT-11 and 0.05 μM CPT. These results are consistent with previously published results regarding the effects of CPT-11 and CPT on intact mouse leukemia (P388) cell DNA synthesis [24]. Also, we found that approximately 2 μM VP-16 inhibited intact MDA MB-468 cell DNA synthesis by 50% (Figure 1); which is consistent with findings reported by Tadou et al. regarding the inhibitory effects of VP-16 on intact mouse splenocyte DNA replication [25]. The presence of low levels of DNA synthesis at higher concentrations of CPT-11, CPT and VP-16 suggests that both topoisomerases I and II may substitute for the functions normally provided by the other [5, 25].

To determine whether SN-38, the active metabolite of CPT-11, and VP-16 inhibit DNA synthesome-mediated *in vitro* DNA synthesis, we performed *in vitro* DNA replication assays (Materials and Methods) in the presence of increasing concentrations of these agents. Additionally, as a positive control, we performed *in vitro* DNA replication assays in the presence of increasing concentrations of CPT, as we have previously demonstrated that this agent strongly inhibits DNA synthesis mediated by the HeLa cell synthesome [Coll et al., 1996]. We found that 0.2 μM SN-38 and 0.5 μM CPT inhibited the incorporation of [^{32}P]-dCMP into newly synthesized

DNA by 50% (Table 1). In addition, we found that approximately 0.5 μM VP-16 inhibited *in vitro* DNA synthesis by 50% (Table 1). These IC_{50} values for *in vitro* DNA replication mediated by the DNA synthesome correlate closely with those concentrations of CPT-11 (SN-38), CPT and VP-16 inhibiting MDA MB-468 intact cell DNA synthesis by 50%. Overall, these results are consistent with the hypothesis that the DNA synthesome represents the intact breast cell's DNA replication machinery.

Effects of SN-38 and VP-16 on DNA synthesome-associated topoisomerase I and II activities

We performed topoisomerase I assays to demonstrate that the topoisomerase I activity present in the DNA synthesome is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA to a relaxed, open-circular form II DNA. As shown in Figure 2 (lanes 7 and 8), the pattern of topoisomers produced by the DNA synthesome-associated topoisomerase I is indistinguishable from that generated by the purified enzyme. Furthermore, we estimated that the level of topoisomerase I present in the Q-Sepharose peak is approximately 1 unit/8 μg of synthesome protein (Materials and Methods).

In order to examine the effects of SN-38, as well as CPT, on DNA synthesome-associated topoisomerase I activity, we performed topoisomerase I assays in the presence of increasing concentrations of these agents (Materials and Methods). In these assays, the inhibition of topoisomerase I activity by SN-38 and CPT results in the accumulation of form I DNA. We observed an extensive level of inhibition of the DNA synthesome-associated topoisomerase I activity by SN-38 and CPT each (Figure 2A, lanes 1-5 and Figure 2B, lanes 1-4; as little as 0.5 μM of either agent caused a significant retention of form I DNA. From these experiments, we determined that 0.5 μM SN-38 and CPT inhibited DNA synthesome-associated topoisomerase I activity approximately 50% (Materials and Methods). These concentrations of SN-38 and CPT

are comparable to those which inhibit both *in vitro* and intact cell DNA synthesis by 50%; supporting the premise that the inhibition of DNA synthesis by SN-38 and CPT results from the inhibition of topoisomerase I activity by these agents.

We also performed topoisomerase II assays to demonstrate that the DNA synthesome contains a fully functional topoisomerase II. Similar to the purified topoisomerase II enzyme, the DNA synthesome-associated topoisomerase II is fully able to decatenate kinetoplast DNA to generate monomeric, open-circular DNAs in an ATP-dependent reaction (Figure 3, lane 3). Furthermore, the DNA synthesome contained in the Q-Sepharose peak is devoid of nuclease contamination as it did not support the relaxation of kinetoplast DNA to the linear DNA fragments (Figure 3, lane 3). We estimated that the level of topoisomerase II present in the Q-Sepharose peak is approximately 1 unit/8 μg of synthesome protein (Materials and Methods).

We next examined the effects of increasing concentrations of VP-16 on DNA synthesome-associated topoisomerase II activity. At 0.5 μM VP-16, the DNA synthesome-associated topoisomerase II was inhibited approximately 50% from supporting the decatenation of kinetoplast DNA networks to open-circular, monomeric DNA (Figure 3, lane 4; Materials and Methods). This concentration of VP-16 is similar to those which inhibit intact cell and *in vitro* DNA replication by 50%; supporting the premise that the inhibition of DNA synthesis by VP-16 results from the inhibition of topoisomerase II activity by the drug.

DNA Topoisomerase I and II Cleavable Complex Stabilization by SN-38 and VP-16

SN-38 interferes with the DNA breakage-reunion activity of topoisomerase I by trapping the enzyme in a reversible ternary (drug-enzyme-DNA) cleavable complex [24, 27]. In this state, topoisomerase I remains covalently attached to the 3' ends of nicked substrate DNA, unable to catalyze the religation of DNA single-strand breaks [28]. It has been shown that cleavable complexes can be selectively precipitated by a strong protein denaturant [27, 29]. Therefore,

utilizing 3' end-labeled substrate DNA, the relative amount of topoisomerase I-DNA cleavable complexes stabilized by SN-38 can be quantified by determining the amount of radiolabeled DNA precipitated by SDS. We performed SDS-precipitation assays using the Q-Sepharose peak fraction to determine the relative amount of DNA synthesome associated-topoisomerase I cleavable complexes stabilized by SN-38. We found that the level of cleavable complexes formed by SN-38 reached a plateau at 5 μM (Figure 4); suggesting saturation. Similar results were also obtained with CPT (Figure 4). The production of significant levels of cleavable complexes at low concentrations of SN-38 and CPT (0.05 μM and 0.5 μM) is consistent with our results demonstrating that 0.2 μM SN-38 and 0.5 μM CPT strongly inhibit *in vitro* DNA synthesis.

Similar to SN-38 and CPT, VP-16 interferes with the breakage-reunion reaction of its target enzyme by trapping topoisomerase II in a reversible ternary cleavable complex [7]. In this state, topoisomerase II remains covalently attached to the 5' ends of broken DNA strands, unable to catalyze the religation of DNA double-strand breaks. Using 3' end-labeled substrate DNA, we determined the relative amount of DNA synthesome-associated topoisomerase II cleavable complexes stabilized by VP-16. We observed that the relative number of cleavable complexes formed by VP-16 peaked at 0.5 μM (Figure 4). The production of significant levels of cleavable complexes at low concentrations of VP-16 (0.05 μM and 0.5 μM) is consistent with our data demonstrating that just 0.5 μM VP-16 strongly inhibits *in vitro DNA* synthesis (Figure 4).

Neutral and Alkaline Agarose Gel Electrophoretic Analyses of the DNA Products Synthesized by the DNA Synthesome in the Presence of SN-38 and VP-16

In order to more closely examine the effects of SN-38, as well as CPT, and VP-16 on DNA synthesome-mediated *in vitro* DNA replication, we subjected the DNA products synthesized by the complex in the presence of these agents to alkaline agarose gel electrophoresis. The DNA products synthesized by the DNA synthesome in positive control

reactions ranged in size from less than 100 nucleotides to two full-length products (ssl and ccc) (Figure 5, lanes 4 and 5). Moreover, resolution of these daughter DNA molecules on neutral agarose gels demonstrates that the majority of these products consist of monomeric form I DNAs as well as topological and replicative intermediates that are resistant to digestion by *Dpn*I (Figure 5, lanes I and 2); which is consistent with the criteria for semi-conservative DNA replication *in vitro*. Additionally, DNA synthesome-mediated *in vitro* replication is large T-antigen dependent, as no DNA products were formed in the absence of the viral protein (Figure 5, lane 3). In the presence of 0.2 μM SN-38 or 0.5 μM CPT or VP-16, the majority of the DNA molecules produced by the DNA synthesome ranged in size from 100-500 nucleotides only, suggesting that these agents strongly inhibited the elongation of nascent DNAs by the complex (Figure 5, lanes 6-8).

DISCUSSION

Numerous studies have demonstrated that SV40 DNA replication *in vitro, as* mediated by crude cellular extracts or reconstituted with purified proteins, represents a potential model for studying the actions of anticancer agents that target mammalian DNA synthesis [12, 13, 30, 31]. The advantages to the employment of this system include that it can mediate various aspects of the DNA replication process and is dependent upon only one viral protein, the large T-antigen, for DNA synthesis. However, when assessing the mechanism of action of an anticancer agent, the use of crude cellular extracts or individually purified DNA replication proteins to mediate SV40 DNA synthesis *in vitro* poses several drawbacks. First, the use of crude extracts does not permit the study of the interaction of an agent with the DNA replication machinery exclusively. As such, discrepancies in the concentrations of an anticancer agent required to inhibit *in vitro* versus intact cell DNA synthesis have been reported [32]. Second, not all of the proteins and enzymes involved in mammalian DNA synthesis have been identified; so, a DNA replication system reconstituted with purified polypeptides may lack the target-protein of an anticancer agent [13]. Therefore, the employment of an *in vitro* model that represents the intact cell's DNA replication machinery would greatly facilitate the process of evaluating the mechanisms of action of agents that target mammalian DNA synthesis.

In this report, we have presented for the first time data that indicate that the breast cell DNA synthesome represents a novel model for studying the actions of CPT-11 and VP-16. We found a close correlation between the IC_{50} values for the inhibition of intact cell and DNA synthesome-mediated *in vitro* DNA replication by CPT-11, or SN-38, (2.0 and 0.2 μM, respectively) and VP-16 (2.0 and 0.5 μM, respectively) (Figure 1 and Table 1); evidence that the

DNA synthesome represents the intact cell's DNA replication machinery. Additionally, we found that similar concentrations of SN-38 and VP-16, 0.5 μM each, inhibited DNA synthesome-associated topoisomerase I and II activities, respectively, by 50% (Figures 2, lane 5; Figure 3, lane 10). These results support the premise that SN-38 and VP-16 impair nucleic acid synthesis by selectively targeting topoisomerases I and II.

Both SN-38 and VP-16 inhibit the activities of topoisomerases I and II, respectively, by stabilizing covalent enzyme-cleaved DNA complexes that are normal intermediates in the catalytic cycles of the enzymes [28, 33]. In accord with their modes of action, we found that the same concentrations of SN-38 and VP-16 that inhibited DNA synthesome-associated topoisomerase I and II activities by 50% (0.5 μM each), also produced significant levels of cleavable complexes (Figure 4). It has been postulated that the interaction of drug-stabilized topoisomerase I and II DNA-cleavable complexes with advancing replication forks leads to replication fork arrest and irreversible double-strand DNA breaks [4, 7, 34]. Consistent with the theory that drug-stabilized topoisomerase I and II cleavable complexes represent blocks to fork progression, we have demonstrated that SN-38, CPT and VP-16 strongly inhibit the elongation of nascent DNA molecules by the DNA synthesome (Figure 5, lanes 6-8). Our results are consistent with previously published results indicating that the exposure of intact diploid human fibroblasts to CPT and m-amsacrine, a topoisomerase II inhibitor with a mechanism of action similar to that of VP-16, inhibits DNA chain elongation in operating replicons [35].

It is widely accepted that both topoisomerases I and II can function as swivelases during the elongation stage of DNA synthesis [5]. However, several reports have provided evidence that topoisomerase I normally functions as the major swivelase during the elongation stage of DNA

replication [36], while topoisomerase II acts only to segregate daughter DNA molecules during the late stages of DNA synthesis [37, 38]. Our observation that VP-16 caused an accumulation of short DNA products (100-500 nucleotides) during synthesome-mediated *in vitro* DNA replication indicates that topoisomerase II may normally function in the early, as well as the late, stages of DNA synthesis. Our results are in agreement with studies performed by Avemann et al. [34] demonstrating that the exposure of SV40 infected CV-1 cells to VM-26, a topoisomerase II inhibitor with a mechanism of action similar to that of VP-16, induces strand breaks in SV40 DNA at replication forks. A role for topoisomerase II in the early stages of DNA replication is further supported by studies demonstrating that in proliferating cells, the functional enzyme is enriched at the sites of attachment of chromatin loops to the nuclear matrix [39]. At these sites, topoisomerase II may be involved in attaching DNA loops to the nuclear matrix as well as unwinding the DNA double helix to allow access of the replication machinery to DNA.

In conclusion, in this report we have provided strong evidence that the breast cell DNA synthesome represents a novel model for examining the mechanisms of action of CPT-11 and VP-16. Furthermore, we have presented data that strongly suggest that topoisomerase II, in addition to topoisomerase I, may function during DNA chain elongation. We anticipate that future studies with the breast cell DNA synthesome may help elucidate the cytotoxic events that occur downstream of CPT-11 and VP-16 stabilized cleavable complex formation. Ultimately, an improved understanding of the modes of action of CPT-11 and VP-16 may aid the development of improved analogues of these agents for breast cancer treatment.

FIGURE LEGENDS

Figure 1. Effects of CPT-11, CPT and VP-16 on intact MDA MB-468 cell DNA synthesis. MDA MB-468 breast cancer cells ($5x10^4$) were seeded onto 60-mm cell culture plates and incubated overnight at 37°C. The cells were then exposed for one hour to increasing concentrations of either CPT-11 ( ), CPT ( ) or VP-16 ( ) (0.05, 0.5, 5, 50, 500 μM, which were dissolved in DMSO), in the presence of [^{3}H]-thymidine (1 μCi/ml). As a negative control for these assays, cells were treated with DMSO alone. Following the incubation, cells were lysed and the level of DNA synthesis was determined by the isolation and counting of ^{3}H incorporated into acid-insoluble material. Each point represents the average of three independent experiments. Bars represent the standard error of the mean.

Figure 2. Inhibition of DNA synthesome-associated topoisomerase I activity by SN-38 and CPT. The Q-Sepharose peak (8 μg) was incubated with 150 ng of supercoiled pSVO$^+$ DNA for 30 minutes at 37°C in the presence of increasing concentrations of SN-38 or CPT (0.05, 0.5, 5, 50, 500 μM, which were dissolved in DMSO). Reactions containing DMSO alone served as negative controls (Figure 2A, lane 6, Figure 2B, lane 5). The reactions (20 μl) were stopped by the addition of SDS to a final concentration of 1%; and the protein was digested with proteinase K (50 ng/μl) for fifteen minutes at 37°C. Afterward, the DNA topoisomers were resolved by electrophoresis (45 volts) through a 1% agarose gel containing TAE buffer (Materials and Methods). Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) and the topoisomers visualized by illuminating the gels with an ultraviolet light source. Figure 2A, lane 6 shows the position of supercoiled pSVO$^+$ DNA. Lane 7 shows the conversion of supercoiled, form I DNA into relaxed, open-circular form II DNA by the DNA synthesome contained in the Q-Sepharose peak. Figure 2A, lanes 1-5 and Figure 2B, lanes 1-4 show the inhibitory effects of SN-38 and CPT, respectively, on DNA synthesome-associated topoisomerase I activity. Figure 2A, lane 7 and Figure 2B, lane 6 show the relaxation of supercoiled pSVO$^+$ DNA to open-circular, form II DNA by 2 units of purified topoisomerase I.

Figure 3. Inhibition of DNA synthesome-associated topoisomerase II activity by VP-16. The Q-Sepharose peak (8 μg) was incubated with 260 ng of K DNA for 45 minutes at 37°C in the presence of increasing concentrations of VP-16 (0.05, 0.5, 5, 50, 500 μM, which were dissolved in DMSO). Reactions containing DMSO alone served as negative controls (lane 3). The reactions were stopped by the addition of 2 μl 0.5 M EDTA and SDS to a final concentration of 1%. After proteinase K digestion (50 ng/μl) and the addition of gel loading buffer to the samples, the DNA products were extracted once with chloroform/isoamyl alcohol. The DNA products were resolved at 100 volts on a 1% agarose gel containing TBE buffer (Materials and Methods) and ethidium bromide (0.5 μg/ml). Following electrophoresis, the DNA products were visualized by illuminating the gel with an ultraviolet light source. Lanes 1 and 2 show the positions of linear and decatenated marker DNA. Lane 3 shows the relaxation of K DNA to decatenated monomers by the DNA synthesome associated topoisomerase II. Lanes 4-8 show the concentration dependent inhibition of DNA synthesome-associated topoisomerase II activity by VP-16. Lane 9 shows that K DNA networks remain unresolved following their incubation with the Q-Sepharose peak in a buffer lacking ATP. Lane 10 shows the relaxation of K DNA to decatenated monomers by purified topoisomerase II.

Figure 4. SDS-K^+ precipitation of SN-38, CPT and VP-16 stabilized topoisomerase cleavable complexes. Reaction assays containing 10 μg of the Q-Sepharose peak, 220 ng of 3'-end labeled $pSVO^+$ DNA and increasing concentrations of either SN-38 ( ), CPT ( ) or VP-16 ( ) (0.05-5 μM), dissolved in DMSO, were incubated for 30 minutes at 37°C. Reactions containing DMSO alone served as controls. The reactions (30 μl) were stopped by the addition of 3 μl 10% SDS and 250 μl of buffer A1 (Materials and Methods). Following their precipitation by KCl, the topoisomerase-DNA covalent cleavable complexes were isolated by vacuum filtration onto glass fiber filters, and the filters were washed and counted in a liquid scintillation spectrophotometer.

Each point represents the average of two independent experiments; bars represent the standard error of the mean.

Figure 5. Neutral and alkaline agarose gel electrophoretic analyses of the DNA products synthesized by the DNA synthesome in the presence of SN-38, CPT and VP-16. *In vitro* DNA replication reactions (25 μl) containing the Q-Sepharose peak (15 μg), $pSVO^+$ DNA (50 ng), T-antigen and either DMSO (positive control), 0.2 μM SN-38, or 0.5 μM CPT or VP-16 were incubated for 1 hr at 37°C. The newly synthesized daughter DNA molecules were isolated and resolved by neutral or alkaline agarose gel electrophoresis (Materials and Methods). **A.** For neutral agarose gel electrophoresis, the position of form I, supercoiled DNA is indicated (lane 1). Lane 2 shows that *Dpn*I-resistant form I, II and III DNA as well as replicative and topological intermediates are produced by the DNA synthesome during *in vitro* replication reactions. Lane 3 shows that the synthesis of daughter DNA molecules by the DNA synthesome is T-antigen dependent. **B.** For alkaline agarose gel electrophoresis, the positions of double-stranded, covalently-closed circular and full-length, single-stranded linear DNA are shown. Size markers for the alkaline gels were derived from a *Hind* III digest of lambda DNA (New England Biolabs) labeled with T4 DNA polymerase by standard methods [22]. Lane 1 shows the DNA products synthesized *in vitro* by the DNA synthesome in the presence of DMSO (positive control); lanes 2-4 show the inhibitory effects of SN-38, CPT and VP-16, respectively, on DNA synthesome-mediated *in vitro* DNA replication.

REFERENCES

1. Nitiss, J.L. (1994) Roles of DNA topoisomerases in chromosomal replication and segregation. Adv. Pharmacol. **29A**:103-132.

2. Champoux, J.J. (1994) Mechanism of catalysis by eukaryotic DNA topoisomerase I. Adv. Pharmacol. **29A**:71-84.

3. Dean, F.B., Bullock, P., Murakami, Y., Wobbe, C.R., Weissback, L. and Hurwitz, J. (1987) Simian virus 40 (SV40) DNA replication *in vitro*: SV40 large T-antigen unwinds DNA containing the SV40 origin of replication. PNAS **84**:16-20.

4. Snapka, R.M. (1986) Topoisomerases can selectively interfere with different stages of simian virus 40 DNA replication. Mol. Cell. Biol. **6**:4221-4227.

5. Yang, L., Wold, M.S., Li, J.L., Kelly, T.J. and Liu, L.F. (1987) Roles of DNA topoisomerases in simian virus 40 DNA replication *in vitro*. PNAS **84**:950-954.

6. Andersen, A.H., Svejstrup, J.Q. and Westgaard, O. (1994) The DNA binding, cleavage and religation reactions of eukaryotic topoisomerases I and II. Adv. Pharmacol. **29A**: 83-101.

7. Liu, L.F. (1989) DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem. **58**:351-375.

8. Schneider, E., Hsiang, Y.H., and Liu, L.F. (1990) DNA topoisomerases as anticancer drug targets. Adv. in Pharm. **21**:149-173.

9. Hsiang, Y-H and Liu, L.F. (1989) Evidence for the reversibility of cellular DNA lesions induced by mammalian topoisomerase II poisons. JBC **264**:9713-9715.

10. Hsiang, Y-H, Lihou, M.G. and Liu, L.F. (1989) Arrest of replication forks by drug-stabilized topoisomerase I cleavable complexes as a mechanism of cell-killing by camptothecin. Cancer Res. **49**:5077-5082.

11. Zhang, H., D'Arpa, P. and Liu, L.F. (1990) A model for tumor cell killing by topoisomerase poisons. Cancer Cells **2**:23-27.

12. Snapka, R.M. and Permana, P.A. (1993) SV40 DNA replication intermediates: analysis of drugs which target mammalian DNA replication. BioEssays **15**:121-127.

13. Malkas, L.H. (1996) SV40 *in vitro* DNA replication: a potential model for studying anticancer drug action. In: The SV40 Replicon Model for Analysis of Anticancer Drugs. RG Landes Co.

14. Hickey, R.J., Malkas, L.H., Pedersen, N., Li, C. and Baril, E.F. (1988) Multienzyme complex for DNA replication in HeLa cells. Moses, R. and Summers, W. (eds.) In: DNA Replication and Mutagenesis, Amer. Soc. Microbiol. Public., Washington D.C., pp. 41-54.

15. Malkas, L.H., Hickey, R.J., Li, C.J., Pedersen, N. and Baril, E.F. (1990) A 21S enzyme complex from HeLa cells that functions in simian virus 40 (SV40) DNA replication *in vitro*. Biochem. **29**: 6362-6374.

16. Applegren, N., Hickey, R.J., Kleinschmidt, A.M., Zhou, Q., Coll, J., Wills, P., Swaby, R., Wei, Y., Quan, J.Y., Lee, M. and Malkas, L.H. (1995) Further characterization of the human cell multiprotein DNA replication complex (MRC). J. Cell. Biochem. **59**:91-107.

17. Wu, Y., Hickey, R.J., Lawlor, K., Wills. P., Yu, F., Ozer, H., Starr, R., Quan, J.Y., Lee, M. and Malkas, L.H. (1994) A 17S multiprotein form of murine cell DNA polymerase mediates polyoma virus DNA replication *in vitro*. J. Cell. Biochem. **54**: 32-46.

18. Coll, J.M., Weeks, J.G., Hickey, R.J., Schnaper, L., Yue, W., Brodie, A.M.H., Syvaoja, J. and Malkas, L.H. (1996) The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. Oncol. Res. **8**:435-447.

19. Malkas, L.H. and Hickey, R.J. (1997) Mammalian cell DNA replication. In: Critical Reviews in Eukaryotic Gene Expression, **7**:125-157.

20. DePamphilis, M.L. and Bradley, M.K. (1986) Replication of SV40 and Polyoma Virus Chromosomes. Salzman, N.P. (ed.) In: "The Papovaviridae Volume I: The Polyomavirus" pp. 99-246, Plenum Press, New York, NY.

21. Tom, T., Malkas, LH. and Hickey, R.J. (1995) Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen dependent SV40 DNA replication activity. J. Cell. Biochem. **63**:259-267.

22. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) In: Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. E.1-E.39.

23. Stillman, B., Gerard, R.D., Guggenheimer, R.A. and Gluzman, Y. (1985) T-antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. **4**: 2933-2939.

24. Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H. and Sato, K. (1991) Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res. **51**: 4187-4191.

25. Tadou, G., Portemer, C., Jaxel, C. and Duguet, M. (1993) Inhibition of DNA synthesis and DNA fragmentation in stimulated splenocytes by the concerted action of topoisomerase I and II poisons. Biochem. Pharmacol. **45**:331-337.

26. Coll, J., Hickey, R.J., Wei, Y. and Malkas, L.H. (1996) The human cell multiprotein DNA replication complex (MRC): The effect of camptothecin on its ability to support *in vitro* DNA synthesis. Cancer Chemother. Pharmacol. **39**:97-102.

27. Hsiang, Y.H. and Liu, L.F. (1988) Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. **48**: 1722-1726.

28. Cremeers, G.J., Lund, B. and Verweij, J. (1994) Topoisomerase I inhibitors: topotecan and irenotecan. Cancer Treat. Rev. **20**:73-96.

29. Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. **260**: 14873-14878.

30. Yamaki, H., Ariga, H. and Tanaka, N. (1986) Inhibition of SV40 DNA replication *in vitro* by 1-N-acyl-3'-N-(trifluoroacetyl)kanamycin. Biochem. Biophys. Res. Commun. **136**:322-328.

31. Tsao, Y-P, Russo, A., Nyamuswa, G., Silber, R. and Liu, L.F. (1993) Interaction between replication forks and topoisomerase I-DNA cleavable complexes: studies in a cell-free SV40 DNA replication system. Cancer Res. **53**:5908-5914.

32. Wobbe, R.C., Dean, F., Weissbach, L. and Hurwitz, J. (1985) *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. PNAS **82**: 5710-5714.

33. Osheroff, N., Corbett, A. and Robinson, M.J. (1994) Mechanism of action of topoisomerase II-targeted antineoplastic agents. Adv. Pharmacol. **29B**:105-124.

34. Avemann, K., Knippers, R., Koller, T. and Sogo, J.M. (1988) Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol. Cell. Biol. **8**:3026-3034.

35. Kauffman, W.K., Boyer, J.C., Estabrooks, L.L. and Wilson, S.J. (1991) Inhibition of replicon initiation in human cells following stabilization of topoisomerase-DNA cleavable complexes. Mol. Cell. Biol. **11**:3711-3718.

36. Kim, R.A. and Wang, J.C. (1989) J. Mol. Biol. **208**:257-267.

37. Ishimi, Y., Sugasawa, K., Hanaoaka, F., Eki, T. and Hurwitz, J. (1992) Topoisomerase II plays an essential role as a swivelase in the late stage of SV40 chromosome replication *in vitro*. JBC **267**:462-466.

38. Ishimi. Y., Ishida, R. And Andoh, T. (1992) Effect of ICRF-193, a novel DNA topoisomerase II inhibitor, on simian virus 40 DNA and chromosome replication *in vitro*. Mol. Cell. Biol. **12**:4007-4014.

39. Gasser and Laemmli (1986) .

Figure 1

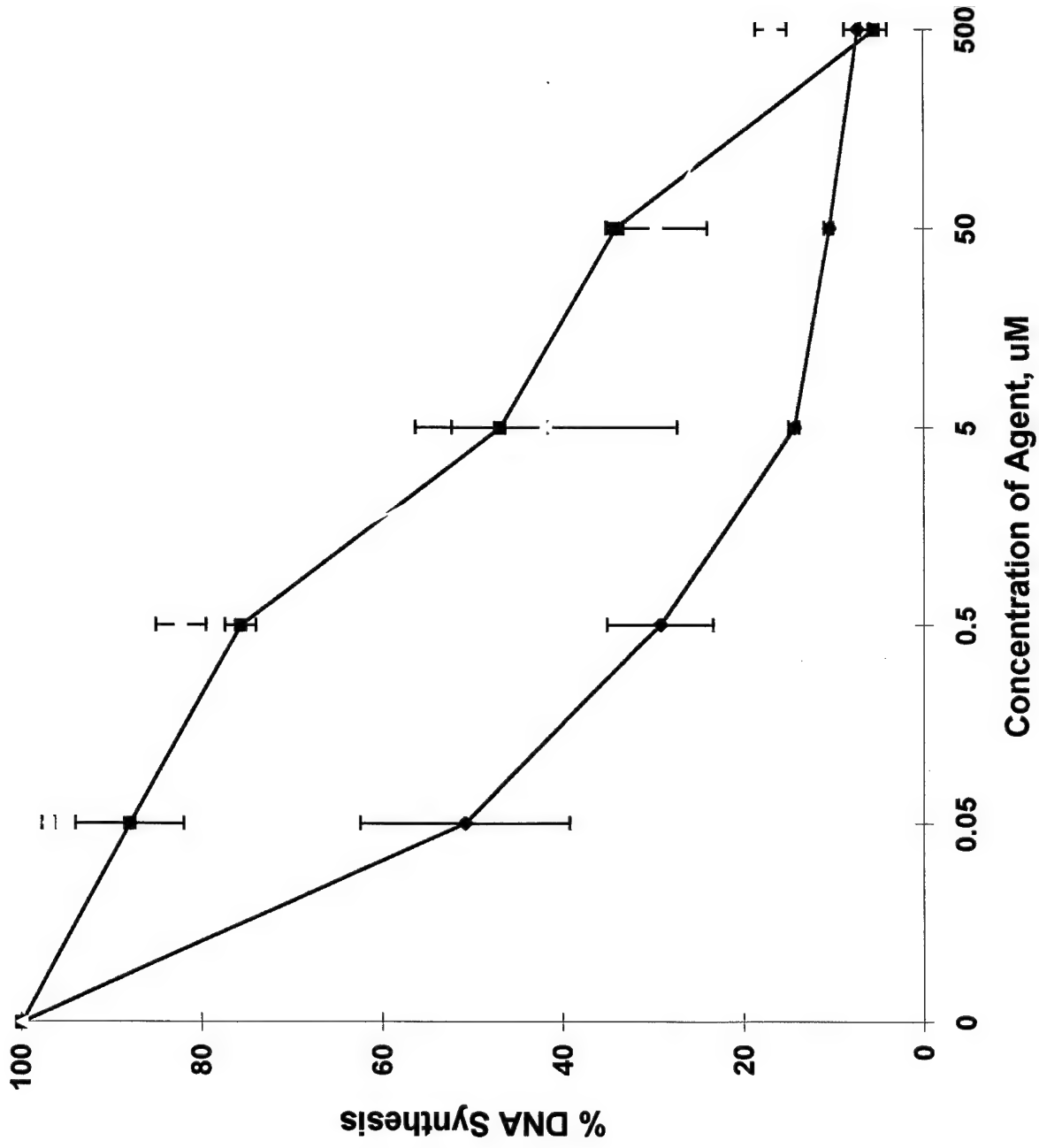

% DNA Synthesis
100
80
60
40
20
0
0
0.05
0.5
5
50
500
Concentration of Agent, uM

Figure 2A

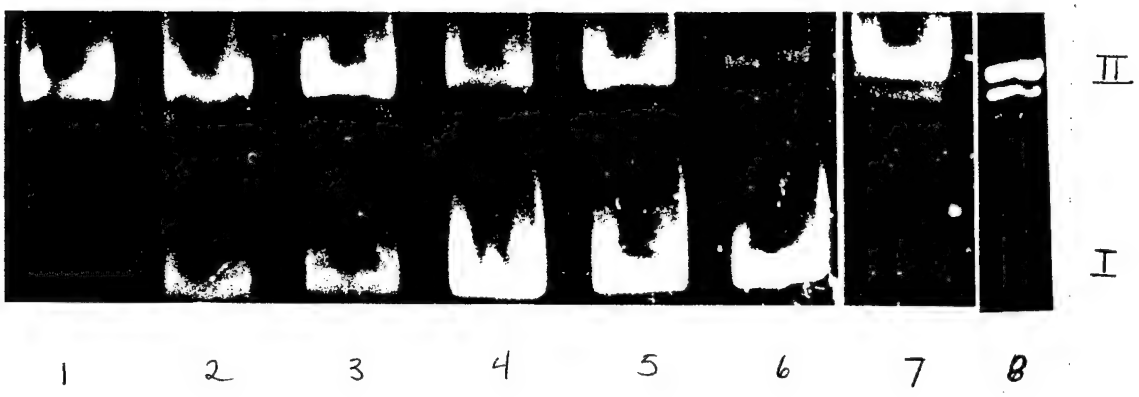


Figure 2B

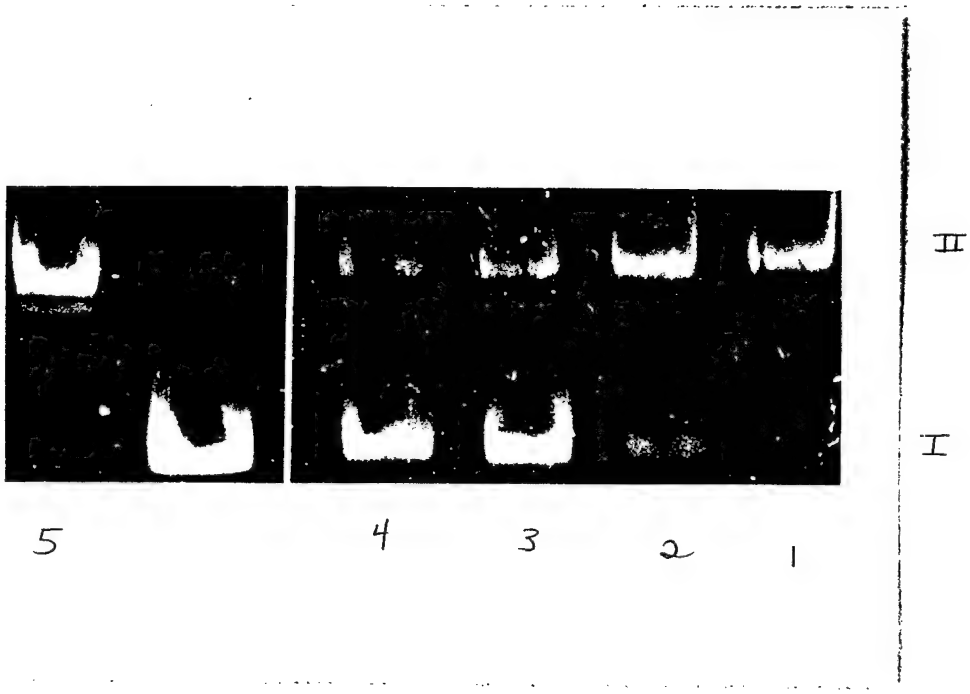


Figure 3

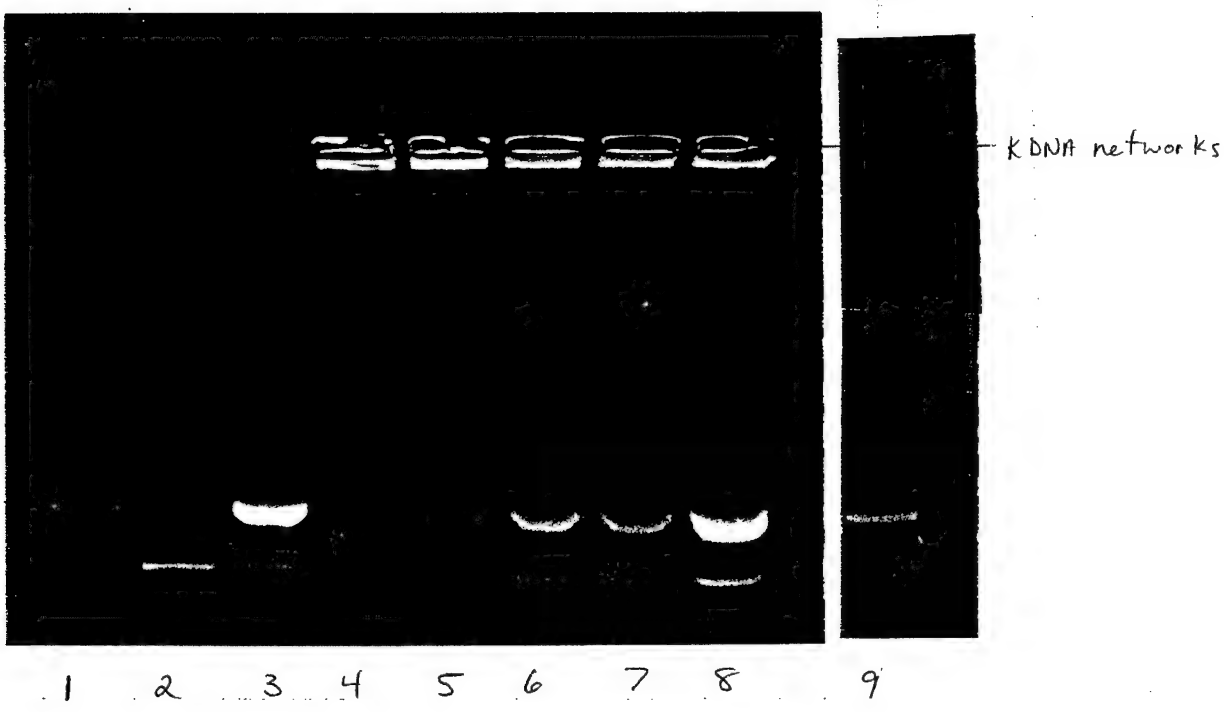


Figure 4

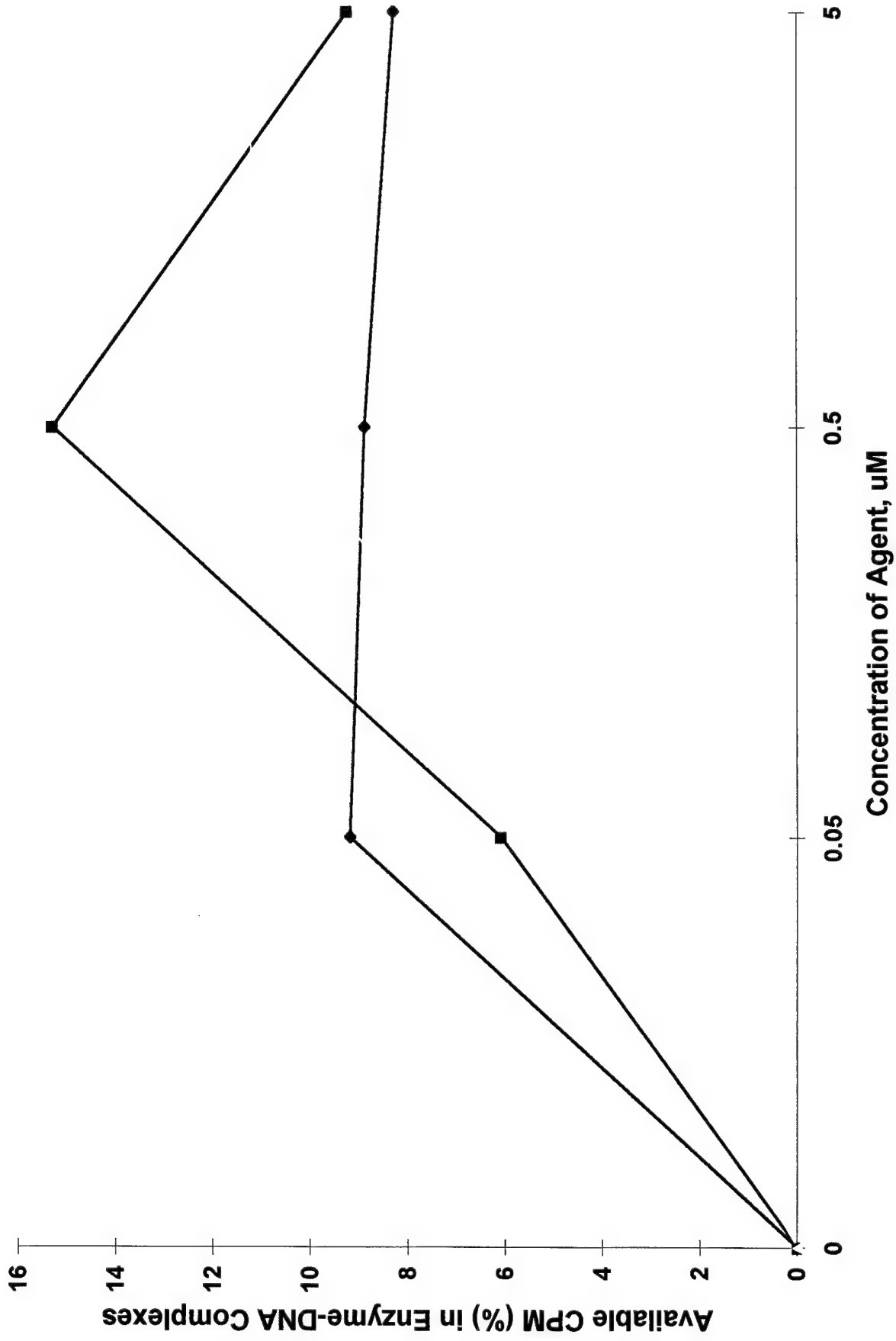

Available CPM (%) in Enzyme-DNA Complexes
16
14
12
10
8
6
4
2
0
0
0.05
0.5
5
Concentration of Agent, uM

Figure 5A

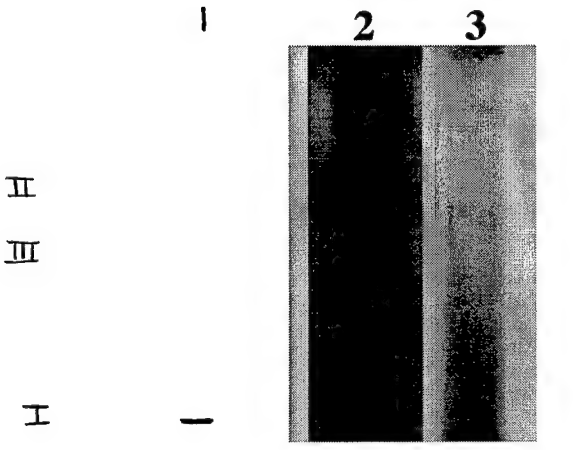


Figure 5B

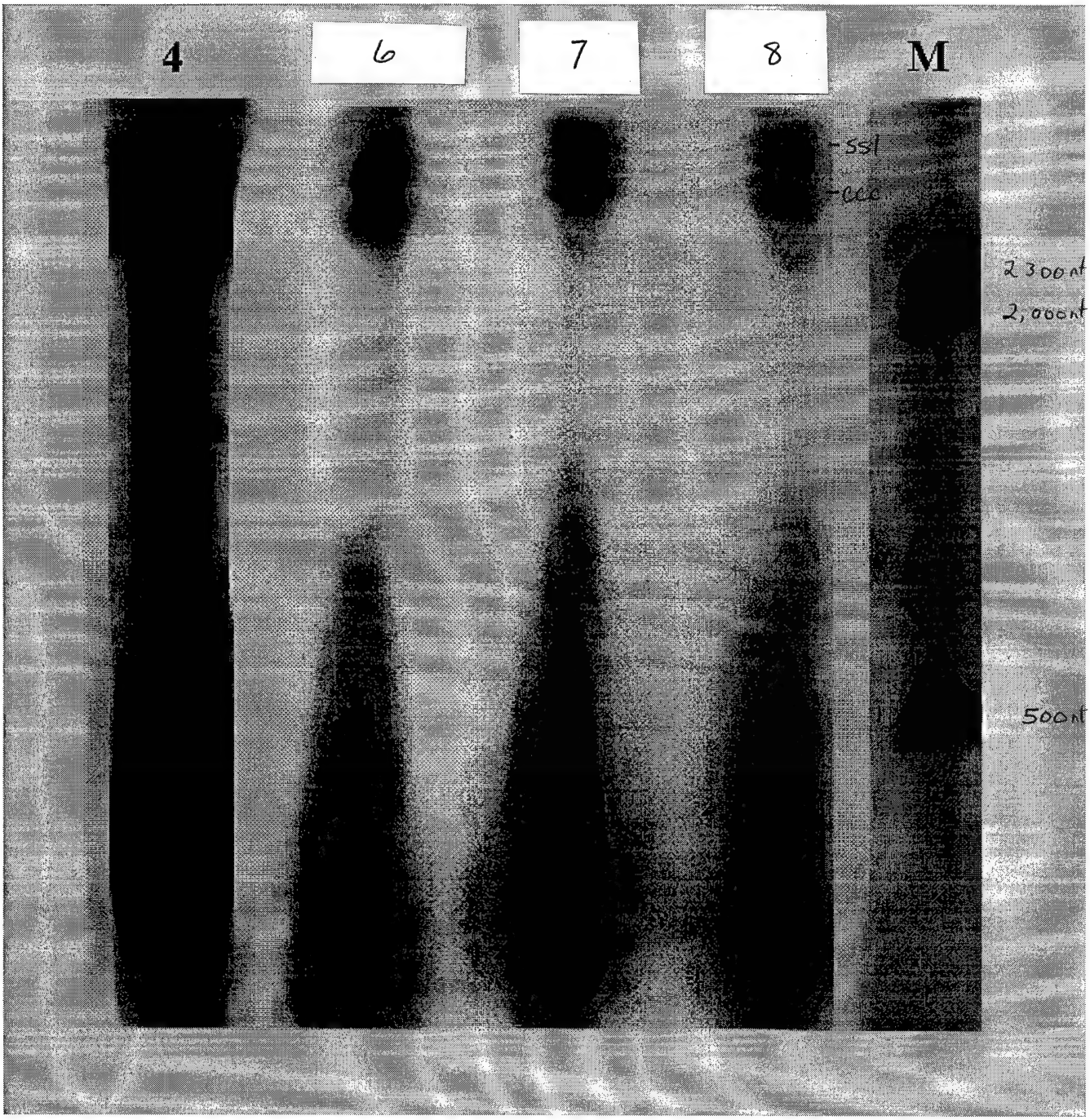


Table 1. IC_{50} values for the inhibition of DNA synthesome mediated *in vitro* DNA synthesis by SN-38, CPT and VP-16.

Anticancer Agent	IC_{50} for *in vitro* DNA synthesis*
SN-38	**0.2 μM**
CPT	**0.5 μM**
VP-16	**0.5 μM**

* *In vitro* DNA replication assays were performed in the presence of increasing concentrations of either SN-38, CPT or VP-16 (0.05-50 μM) as described in the Materials and Methods. IC_{50}s were determined from the standard curves generated from these assays.

revised July 31, 1997 DAMD94J4157

Curriculum Vitae

Jennifer M. Coll

4809 Roland Avenue
Apartment 3A
Baltimore, MD 21210
(410) 706-2313 (W)
jcoll@umabnet.ab.umd.edu

Education

University of Maryland at Baltimore *1993-present*
Baltimore, Maryland
PhD candidate, Department of Pharmacology and Experimental Therapeutics

University of Notre Dame *1988-1992*
Notre Dame, Indiana
B.S., Biological sciences

Research Experience

Doctoral Research
Characterization of a multiprotein complex for DNA replication, designated the DNA synthesome, from human breast cancer cells and breast tumor tissue. Examining the mechanisms of action of the DNA topoisomerase I and II inhibitors, camptothecin and etoposide, respectively, on DNA synthesome mediated *in vitro* DNA replication.

Research Interests

- Mechanisms and regulation of DNA replication in both non-malignant and malignant breast cells
- Mechanisms of action of anti-breast cancer chemotherapeutic agents that target the cellular DNA synthetic process
- Hormonal regulation of DNA synthesis in breast cancer cells

Awards and Recognition

- United States Army Medical Research and Development Command Breast Cancer Predoctoral Fellowship, 1994-present
- Who's Who Among U.S. Graduate Students, 1996
- The National Dean's List, 1996
- Invited Participant to the American Association for Cancer Research sponsored *Histopathobiology of Cancer Workshop*, Keystone, CO, July, 1995
- University of Maryland Cancer Center Travel Award, 1996
- University of Maryland Graduate Student Travel Award, 1995
- Sigma Xi Scientific Society Grant-in-Aid of Research, 1994

Professional Memberships

- Associate Member of the American Association for Cancer Research, 1995-present

- Sigma Xi, The Scientific Research Society, 1996-present
- Federation of American Societies for Experimental Biology (FASEB), 1996-present
- Women in Cancer Research, 1996-present
- Association for Women in Science, 1994-present
- Breast Cancer Coalition, 1996-present
- Department of Pharmacology and Experimental Therapeutics representative to the University of Maryland Graduate Student Government Association, 1993

Mentoring Experience

- Research advisor to Erica A. Cronkey, Masters student, Department of Pharmacology and Experimental Therapeutics, UMAB
- Research advisor to Rosemin Daya, Medical and Technology Research student, UMAB

Inventions

- Mini-fractionation scheme for the purification of the DNA synthesome from tissue samples, 1997 (UMAB disclosure #)

Abstracts

- **J. Coll**, Y. Wei and L.H. Malkas, A unique model to investigate the mechanisms of action of camptothecin on DNA synthesis. Presented at the 1994 American Society of Biochemistry and Molecular Biology Meeting held in Washington, D.C.

- L. Malkas, Y. Wu, N. Applegren, N. Li, **J. Coll**, N. Tuteja and R.J. Hickey, The mammalian cell multiprotein DNA replication complex (MRC). Presented at the 1994 American Society of Biochemistry and Molecular Biology Meeting held in Washington, D.C.

- **J. Coll**, J. Glazer, R.J. Hickey and L.H. Malkas, Multiprotein replication complex protein-protein interactions. Presented at the 1994 McGill University Conference on the Regulation of Eukaryotic DNA Replication held in Montreal, Quebec, Canada.

- **J. Coll**, R.J. Hickey, Y. Wei and L.H. Malkas, The multiprotein DNA replication complex (MRC): The effect of camptothecin on its ability to support DNA synthesis *in vitro*. Presented at the 1996 New York Academy of Sciences Meeting on Camptothecins held in Bethesda, MD.

- **J. Coll**, R.J. Hickey, J. Weeks-Sekowski, L. Schnaper, W. Yue, A.H.M. Brodie and L.H. Malkas, Human breast cancer cells mediated DNA replication via an organized DNA synthesome. Presented at the 1996 Annual Meeting of the American Association for Cancer Research held in Washington, D.C.

- P. Bechtel, **J. Coll**, L. Malkas and Hicley, R.J., The identification of structural alterations in the DNA synthetic apparatus of human breast cancer cells. Presented at the 1996 Annual Meeting of the American Association for Cancer Research held in Washington, D.C.

- **J. Coll**, Y. Wei, R.J. Hickey and L.H. Malkas, Protein-Protein interactions within the human breast cell DNA synthesome. Presented at the 1996 McGill University Conference on the Regulation of Eukaryotic DNA Replication held in Quebec, Canada.

- **J. Coll**, R.J. Hickey, L. Schnaper, Y. Wei and L.H. Malkas, Protein-Protein interactions within the human breast cell DNA synthesome. Presented at the 1996 Annual San Antonio Breast Cancer Symposium held in San Antonio, Texas.
- **J. Coll**, E. Cronkey, R.J. Hickey, L. Schnaper and L.H. Malkas, The human breast cell DNA synthesome: the effects of irenotecan and etoposide on its ability to support *in vitro* DNA synthesis. Presented at the Annual 1997 Meeting of the American Association for Cancer Research held in San Diego, CA.

• C. Simbulan-Rosenthal, D. Rosenthal, R.J. Hickey, L.H. Malkas, **J. Coll** and M. Smulson, Poly(ADP-ribose) polymerase (PARP) is required for expression or assembly of components of the multiprotein DNA replication complex (MRC). Presented at the 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology.

• **J. Coll**, E.A. Cronkey, R.J. Hickey, L. Schnaper and L.H. Malkas, The breast cell DNA synthesome: a novel model for examining the mechanisms of action of irenotecan and etoposide. To be presented at the 1997 U.S. Army Medical Research and Development Command Era of Hope Breast Cancer Research Meeting.

Platform Presentations

• Protein-Protein interactions within the breast cancer cell DNA synthesome **(J. Coll)**. Presented at the 1997 Annual Graduate Student Research Day, UMAB.

Manuscripts

• N. Applegren, R.J. Hickey, A.M. Kleinschmidt, Q. Zhou, **J.M. Coll**, P. Wills, R. Swaby, Y. Wei, J.Y. Quan, M.Y.W.T. Lee and L.H. Malkas (1995). Further Characterization of the Human Cell Multiprotein DNA Replication Complex. J. Cell. Biochem. 59: 91-107.

• **J.M. Coll**, R. J. Hickey, Y. Wei and L.H. Malkas (1996). The Human Cell Multiprotein DNA Replication Complex (MRC): The Effect of Camptothecin on its Ability to Support *in vitro* DNA synthesis. Cancer Chem. Pharmacol. 39: 97-102.

• **J.M. Coll**, J.W. Sekowski, R.J. Hickey, L. Schnaper, W. Yue, A.M.H. Brodie, L. Uitto, J.E. Syvaoja and L.H. Malkas (1996). The Human Breast Cell DNA Synthesome: Its Purification from Tumor Tissue and Cell Culture. Oncology Res. 8: 435-447.

• **J.M. Coll**, R.J. Hickey, E.A. Cronkey, H.Y. Jiang, L.Schnaper, L. Uitto, J.E. Syvaoja and L.H. Malkas (1997). Mapping Specific Protein-Protein Interactions within the Core Component of the Breast Cell DNA Synthesome. Oncology Res. (submitted).

• **J.M. Coll**, E.A. Cronkey, R.J. Hickey, L. Schnaper, L.H. Malkas (1997) The Breast Cell DNA Synthesome: A Novel Model for Examining the Mechanisms of Action of Irenotecan and Etoposide. Biochemical Pharmacol. (submitted).

Addendum

Figure 1. Effects of CPT-11, CPT and VP-16 on intact MDA MB-468 cell DNA synthesis. CPT-11 (■), CPT (◆) and VP-16 (▲).

Figure 4. SDS-K^+ precipitation of SN-38, CPT and VP-16 stabilized topoisomerase cleavable complexes. SN-38 (▲), CPT (■) and VP-16 (◆).

Figure 5. (B) Lane 1, positive control. Lanes 2-4, in vitro replication products formed in the presence of SN-38, camptothecin and etoposide.